African Journal of Pharmacy and Pharmacology

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Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b;

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African Journal of Pharmacy and Pharmacology

ARTICLES

Review

Historical overview of pharmaceutical education in Bulgaria

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A survey of the pharmaceutical education in Bulgaria is performed, analyzing written records of various origin and content. Written documents are reviewed separately in chronological order. They all seem to lend support to the conclusion that the development of pharmaceutical education is following the historical circumstances. Relatively strong is the impact of West-European pharmaceutical education traditions in the last decades, because of the uniting of Bulgaria to the European Union (EU), following of the EU directive 2005/36/EC on recognition of professional qualification and the Bologna declaration.

Key words: European Union, Bulgaria, pharmaceutical education.

INTRODUCTION

Bulgaria is a new member of the European Union (EU) since 2007. The country is situated in the Southern-East part of Europe. It borders Romania to the north, Serbia and Macedonia to the west, Greece and Turkey to the south, as well as the Black Sea to the east. According to the 2011 census, the population of Bulgaria is 7,364,570 people, down from a peak of 9 million inhabitants in 1989. The literacy rate is 98% as a percentage of people of ages 15 and above (World Bank, 2012). Education is one of the most powerful instruments for reducing poverty and inequality and it lays a foundation for sustained economic growth. The education of health professionals is a challenge in the current climate of shrinking all kind of budgets including educational, increasing morbidity, and specialization of patient care.

The economic, political and social changes in Bulgaria have an important impact on all aspects of the social life in the country as well as on the pharmaceutical activities and pharmaceutical education. Until 1989, the pharmaceutical system was centralized; community pharmacies, hospital pharmacies, wholesalers, pharmaceutical manufacturing and institutes were possession of the state. Pharmacy education has been traditionally oriented towards industry.

After the changes in 1989, the Bulgarian pharmaceutical system has turned to market-oriented. It has been going through privatization. The first Bulgarian law of the drugs and pharmacies in the human medicine was introduced in 1995 and it tried to harmonize the Bulgarian drug regulation with that of the EU. In 2007, new law of the drug products in the human medicine that is harmonized with the EU directives in this sphere was introduced. The new global tendencies in the development of pharmacy education that lead to emphasizing the patient's health and quality of life affect the development of the pharmaceutical education. All these circumstances, together with the increased interest in interdisciplinary and interprofessional education lead to the changes in the education of the pharmacists in Bulgaria (Shugars et al., 1991; Walker et al., 1998; Ryan and Hager, 2001).

BRIEF HISTORICAL OVERVIEW

After the liberation of Bulgaria from the Turkish invasion, during the period 1879 to 1941, only pharmaceutical assistants were graduating in Bulgaria. Their education was carried out in the pharmacies. After a defined probation, they had to cover a state examination. That type of pharmaceutical education was regulated through regulations: the assistant students have to been junior

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high school graduates and have to be fluent in French, German or Russian. First the education was for three years, but in 1982 it was decreased to 2 years (Dimitrova, 1999).

At that time, higher pharmaceutical education could only be achieved abroad in Austria, Germany, France, Switzerland, Romania, Russia, etc. In order to be legally qualified, the students had to qualify from pharmacy programme delivered by a higher education institution with duration of at least 3 years and after that, to pass a year probation in a pharmacy. After that, they had to cover state examinations (Dimitrova, 1999).

Historically, the first Pharmaceutical Faculty in Bulgaria was founded in 1942 as a Pharmaceutical Department at the Faculty of Natural Sciences and Mathematics, Sofia University. On 30 June 1951, with a State Decree No. 325, it was transformed into a separate Pharmaceutical Faculty at the newly established Medical Academy "Valcho Chervenkov" (Figure 1). The first dean of the Faculty of Pharmacy, Sofia, Professor Dimitar Dalev was elected on 10 July 1951 (Dimitrova, 1999; Bulgarian Central State Archive, 2012) (Figure 2). The total number of the educated students in 1951 was 362. There was only one doctoral position granted by that time and it was done on a competitive basis (Figure 3).

In 1995, the Medical Academy was renamed to Medical University with four faculties that include Medicinal, Dental medicine, Pharmaceutical and Faculty of Public Health. Until the establishment of the Pharmaceutical Faculty in Plovdiv (Medical University, Plovdiv) in 2003, the Pharmaceutical Faculty (Sofia) was the only place in Bulgaria that educates pharmacists (http://medun.consultcommerce.bg/?lang_id=1&prm=fac &subprm=farf). Later in 2008, a Faculty of Pharmacy was established at the Medical University, Varna (http://www.muvarna.bg/muVarna/index.php?option=com _content&view=article&id=206&Itemid=61).

The education course is 5 years. The first and second year of the university studies are devoted mainly to Chemical Sciences, Mathematics, Botany and Medical Sciences. Years 3 and 4 focus on Pharmaceutical Technology, Pharmacology, Pharmacognosy, Pharmacoeconomics and Social Pharmacy, year 5 focuses on Pharmaceutical Care, Pharmacotherapy and Medical Sciences. A six months traineeship finishes on the 5th year together with preparation of a Master's thesis and the 4 state exams with which university studies typically end. Industrial Pharmacy and Clinical (hospital) Pharmacy are integrated disciplines at the Faculty of Pharmacy, Sofia, Bulgaria. All the graduated students obtain the degree "Master of Pharmacy". 100 to 120 Bulgarian and 25 to 30 foreign students are accepted for training every year.

In the beginning, during the school year 1942/1943, only two disciplines were taught, Pharmacognosy with Galenic Pharmacy and Pharmaceutical Chemistry. In 1947 a third discipline was established, the Galenic

Pharmacy course was separated thus forming three disciplines.

Nowadays, 6 departments in the Faculty of Pharmacy, Sofia are structured as: (A) Pharmaceutical Technology and Biopharmacy, (B) Pharmacognosy and Pharmaceutical Botany, (C) Pharmaceutical Chemistry, (E) Chemistry, (F) Pharmacology and Toxicology, and (G) Social Pharmacy.

CURRICULUM

Since 1989, many changes in the curriculum of the Faculty of Pharmacy, Sofia have been done in order to harmonize it with the curricula of the other pharmaceutical schools in the EU and to equalize the diplomas according to the EU directive 2005/36/EC on recognition of professional qualification and the Bologna declaration. The EU directives give the basic concept of pharmaceutical education. The educational programs are in accordance with the European standards. The training of the students comprises lectures, seminars and practical laboratory work. Figure 4 gives information about the hour's proportion between the different subject areas and proves the harmonization of the education.

Many new aspects and study areas have been introduced since 1989, such as Biopharmacy, Clinical Laboratory, Biology and many others. Especially in the curricula of the Department of Social Pharmacy, many new study areas such as: History of Pharmacy, Pharmacoeconomy, Social Pharmacy and Pharmaceutical Legislation and few free eligible disciplines like Pharmacoepidemiology, Pharmaceutical Marketing and Paediatric Drug forms have been introduced. The orientation of the pharmacist has changed from the product to the patient. The expansion of the role of the pharmacists received an important boost in 1990, when Helper and Strand coined the term pharmaceutical care (Hepler and Strand, 1990). Pharmaceutical care is the responsible provision of drug therapy for the purpose of achieving definite outcomes that improve the patient's quality of life (Hepler and Strand, 1990). In 2000, a new course in Pharmaceutical Care was introduced first as a free eligible subject and after that, two years later as a regular discipline. The lectures and seminars of this subject are delivered during the first semester of the fifth year of the studies. The lectures cover new communicational skills and brain storming sessions on different pharmacy practice cases. Table 1 lists the MSc Pharmacy Curriculum that was revised in 2007. A complete regular training course in English has been introduced since the academic year 2007/2008.

IMPACT

Since the changes in 1989 in Bulgaria, the changes in the
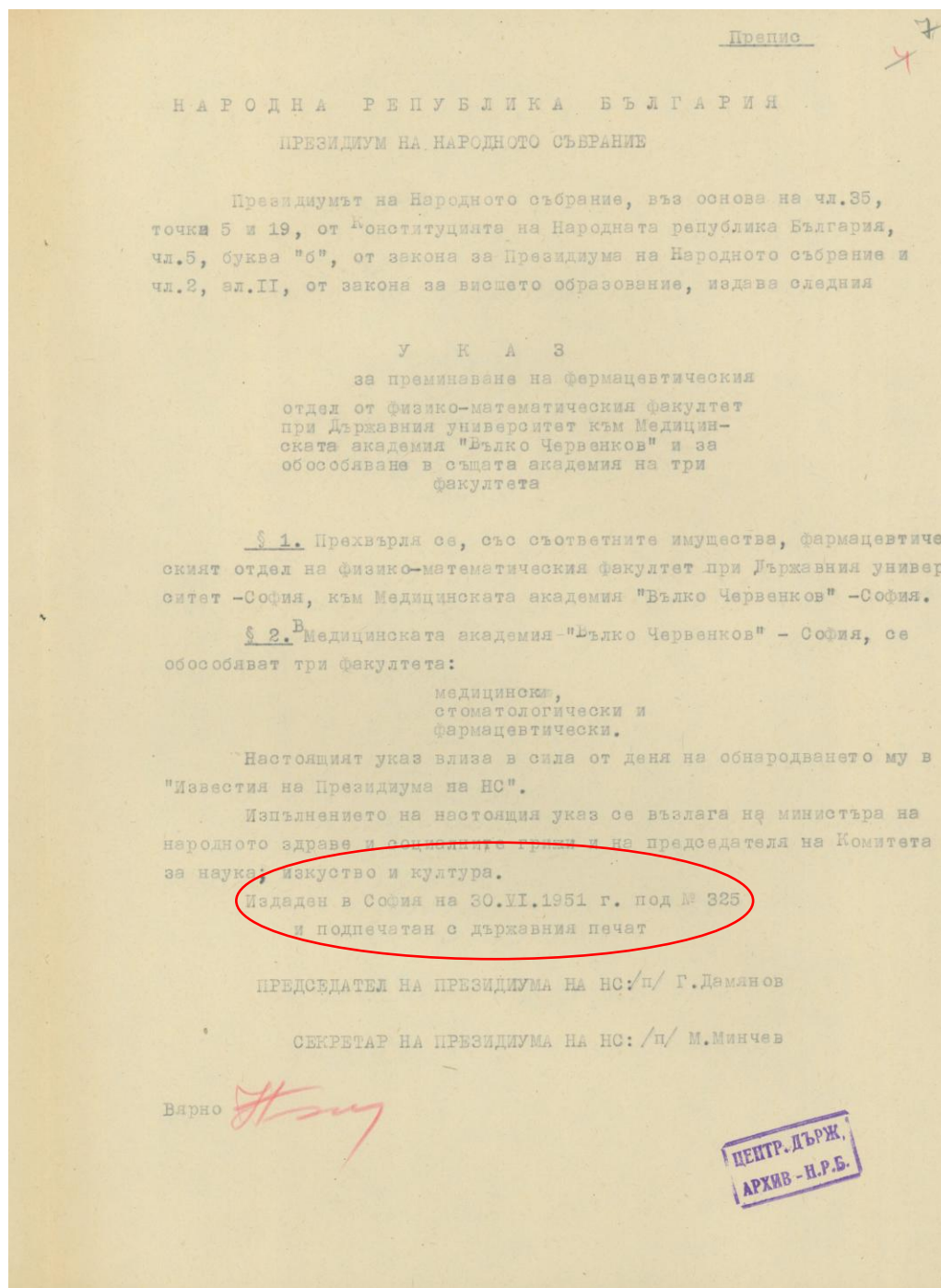
Figure 1. Copy from State Decree for the transfer of the Pharmaceutical Department of the Faculty of Natural Sciences and Mathematics, Sofia University to the Medical Academy "Valcho Chervenkov." Source: Bulgarian Central State Archive (2012).

pharmaceutical education were mandatory in order to preserve the traditions of the profession and to develop modern pharmaceutical specialists. The introduction of the new specialties in the training course such as Clinical Pharmacy, Pharmacoepidemiology and Pharmaceutical Care improves the quality of the services and guarantees a good level of patient care both in community and

hospital pharmacies conditions. On the other hand the course of Pharmacoeconomy gives a possibility for training of highly qualified pharmacists that participate in the process of decision taking of all aspects of pharmaceutical management: from production planning to reimbursement and pricing of the drugs. The impact of all these educational changes are still in process of

Figure 2. Copy from Public Act for entering his duties of the first Dean of the Faculty of Pharmacy, Sofia, Professor Dimitar Dalev.
Source: Bulgarian Central State Archive (2012).

Figure 3. Copy from the Dean's report at the end of the first Faculty of Pharmacy year.
Source: Bulgarian Central State Archive (2012).
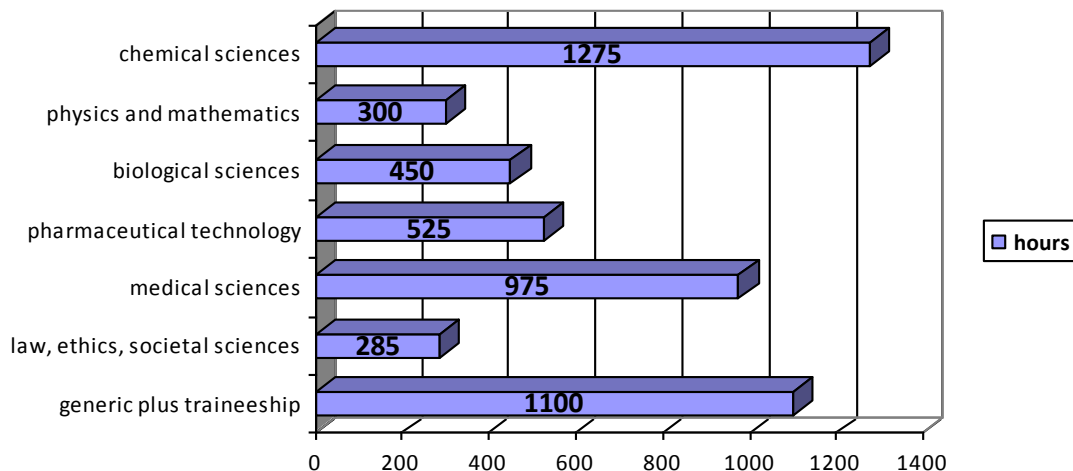
Figure 4. Students' hours by subject area
Source: http://www.pharmine.org/losse_paginas/Country_Profiles/Bulgaria 2012).

Table 1. Outline of the MSc Pharmacy Degree Curriculum in Sofia, Bulgaria.

Course title	Hours of study		Credits
	Theory	Laboratory	
Year 1			
Higher Mathematics	30	30	4
Molecular Biology	30	30	4
History of Pharmacy	30	-	2
Inorganic Chemistry	45	75	8
Physics and Biophysics	60	30	6
Latin	-	60	4
Foreign Language	-	60	4
Sport	-	120	8
Statistics in Pharmacy	15	30	3
Human Anatomy	30	15	3
Year 2			
Human Physiology	60	30	6
Pathoanatomy	15	15	2
Information Technologies	-	30	2
Pathophysiology	30	30	4
Analytical Chemistry	60	120	12
Organic Chemistry	60	120	12
Microbiology and Virology	60	60	8
Year 3			
Physical Chemistry	30	60	6
Botany	60	60	8
Medical Devices	-	30	2
Biochemistry	45	45	6
Pharmaceutical Chemistry	90	135	15
Pharmaceutical Technology Part I	60	150	14
Clinical Chemistry	15	45	4

Table 1. Contd.

Course title	Hours of study		Credits
	Theory	Laboratory	
Year 4			
Pharmacognosy part I	60	150	14
Pharmacology	60	120	12
Social pharmacy and Pharmaceutical Legislation	60	90	10
Hygiene and Ecology	30	15	3
Pharmaceutical Technology Part II	60	165	15
Pharmaceutical Analysis	60	165	15
Pharmacoeconomics	30	45	5
Toxicology	30	60	6
Medical Genetics	15	15	2
Year 5			
Pharmacotherapy	60	90	10
Pharmacognosy, Part II	30	60	6
Biopharmacy and Pharmacokinetics	30	90	8
Bromatology	30	30	4
Pharmaceutical Care	30	45	5
Free elective subjects	30	30	4

Syllabus framed in 2011/2012.

assessment, but the first evaluations are positive, rated both by the pharmaceutical society (Bulgarian pharmaceutical days, 2012 overview) and by the nation (many pharmacists are involved in health state commissions).

Conclusions

Pharmaceutical education in Bulgaria started 70 years ago with the foundation of two departments, Pharmacognosy with Galenic Pharmacy and Pharmaceutical Chemistry. Now, there are 6 separate departments in the Faculty of Pharmacy, Medical University, Sofia. The education curriculum is covering the requirements of the EU directives for harmonization of the education. The new changes in the education lead to changes in the way of thinking of the pharmacists, to improvement of the quality of the pharmaceutical activities in the pharmacy, and to improvement of the communication gap between the patient and the health care providers. The experience of the first Faculty of Pharmacy, Sofia marks the path for development of the newly established Faculties of Pharmacy in Plovdiv and Varna.

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Full Length Research Paper

Antiphospholipid antibodies in acute cardiac attacks

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Cardiovascular diseases have prevailed all over the world in the last few decades. Mortality and morbidity rates due to myocardial infarction (MI) and angina pectoris (AP) increased and affect younger ages in the present days. There are very few studies that have been carried out to define the prevalence of antiphospholipids (APLs) as a marker to help the people at risk in Saudi Arabia. The objective of our study was to examine the antiphospholipid antibodies levels on plasma including anticardiolipin antibody (ACA) and lupus anticoagulants (LA) among Saudi men living in the metropolis of Riyadh, Kingdom of Saudi Arabia. This study was carried out on a sample of 50 Saudi men, aged less than 45 years, who had angina pectoris (AP) and myocardial infarction (MI) going to Riyadh Medical Complex (RMC) and Al-Iman General Hospital from March, 2006 to January, 2008. They were compared with the control group of forty subjects comprising students and staff of the Riyadh College of Health Sciences. Antiphospholipids antibodies can be detected in patients with autoimmune disorders. The primary antiphospholipid antibody syndrome results from reaction of the immune system. In this study, ACA and LA were tested by Enzyme-linked immunosorbent assay (ELISA) and activated partial thromboplastin time (APTT), respectively. The present results showed a significant increase of ACA in 88% of patients. 16 patients (32%) showed positive LA. Antiphospholipids antibodies were found to be significantly associated with MI and in acute attacks of AP especially in patients showing coronary ischemia or thrombosis. The association of LA and ACA showed marked significant correlation when considered together ($P < 0.05$). More attention should be paid by cardiologist to antiphospholipid syndrome, as it is among the severe and fatal diseases. The rise in APLs is a marker for recurrent stroke risk. Further studies in the area are needed.

Key words: Anti phospholipids, myocardial infarction, angina pectoris, Saudi Arabia.

INTRODUCTION

Antiphospholipids syndrome can be classified as a primary or secondary immunological disease which may be associated with other autoimmune diseases. Antiphospholipids syndrome may be among the causes of vascular thrombosis, pregnancy loss and thrombocytopenia with raised levels of antiphospholipids antibodies (Ravelli and Martini, 2005). Acute heart attacks including AP and MI are diseases engendered by several factors that endangered many young lives (Nevas et al., 2001). APLs had been associated to patients with arterial thrombosis including early coronary artery and cerebrovascular thrombosis (Galli et al., 2003). The antiphospholipids syndrome is also associated with arterial and venous thrombosis. Asherson et al. (2003), Renan et al. (2004) and Ruffiatti et al. (2009) have identified three primary classes of antibodies associated with the antiphospholipids antibody syndrome. They are Anticardiolipin, Lupus anticoagulant and Beta-2-Glycoprotein 1.

Zanon et al. (2004) have in their work identified that in APS, there exist a variety of cardiac affections including valvular lesions, myocardial dysfunction, MI. In another study by Girardi et al. (2004), APS is also associated with miscarriages, preterm labor, low birth weight and preeclampsia in 20 to 40% of patient. The processes by which the antiphospholipids antibodies cause thrombophilia have not been established. APLs might inhibit phospholipids-dependent blood coagulation.

According to Triplett (2002) and Ruffatti et al. (2009), APLs could negate the usual anticoagulant protein C thus

cause continuous cell activation leading to enhanced thrombin generation (Cervera et al., 2002; Ruiz-Irastorza et al., 2004; Salmon and Girrardi, 2004).

Going by the work of Nomura et al. (2003), Van Wijk et al. (2003) and Giannakopoulos et al. (2007), thrombosis of coronary artery or its branches were solely of platelet origin or platelet-derived microparticles shed from cell membrane of severely damaged, necrotic or apoptotic cells. Previous studies showed that about 3% of all Cardiac Attack Disease (CAD) cases occurred under age of 45 years (Avcin et al., 2008).

APL is identified increasingly as a main cause of vascular thrombosis in patients; catastrophic anti-phospholipids syndrome should take much care and recognition by cardiologists, being one of the fatal disease (Doaa et al., 2010). The present study was carried out to measure and compare levels of APL in AP and MI patients less than 45 years age and admitted to Coronary Care Unit (CCU), with normal subjects.

MATERIALS AND METHODS

The present study was undertaken at Al-Iman General Hospital (AIGH) and Riyadh Medical Complex (RMC), Riyadh, KSA during the period of March, 2006 to January, 2008. Two groups had been established:

1. Group A Table 1: The patients group; 50 male patients from AIGH and RMC with the age ranging from (20 to 44 years). The selection of patients of AP and MI had the following criteria: (a) Age < 45 years at the initial stage of AP and MI; (b) Complete absence of hypertension, Diabetes and obesity; (c) Confirmed past history of MI in accordance with American Heart Association Guidelines (Lockshin, 2006). Samples from the patients were collected from 8 to 12 weeks after admission to the hospital.
2. Group B Table 2: Negative control group, 40 students and staff-members of Riyadh College of Health Sciences (RCHS), with the age ranging from (24 to 42 years) having no history of MI or AP.

10 ml of venous blood sample was collected in vacutainers containing 0.109 sodium citrate as anticoagulant. The samples were centrifuged at 3500 × g at 4°C for 15 min, then recentrifuge at 2500 × g at 4°C for 15 min to minimize residual platelets at less than 10 × 109/L and then stored at -20°C in aliquots of 300 µl. Of all the patients, none of them was on oral anticoagulents. The serum was collected from patients and control in vacutainers tube without anticoagulant frozen at -20°C for testing for ACA. Standard diluted sera samples of 1:100 was used and added to a coated microplates well with reference standards, then washed twice and incubated with anti-human horseradish peroxidase (HRP) and a substrate at room temperature (20 to 25°C). A color appears after 15 min; ELISA reader at 450 nm was used to measure IgG ACA. A record of IgG ACA equal or above 12 IgG phospholipid (GPL units)/L and IgM ACA above 7 IgM phospholipid (MPL units)/L was taken as significant value.

The condition for positive cases of LA is considered when there is extended coagulation time in stage I or persistent prolongation of time in stage II after adding equal volume of normal plasma. The case can be positive also if there is normal activated partial thromboplastin time reversal due to inhibition of anticoagulant effect on addition of excess phospholipid reagent. Positive cases for LA and ACA were confirmed using both liquid phase coagulation assays and solid phase ELISA assays. APL were positive if it was ≥

12 GPL/units (Miyakis et al., 2006).

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 11 was used for all statistical analysis. Student t-test was applied to compare any quantitative data while the chi-square test was used for qualitative variables. $P < 0.05$ was taken to indicate statistical significance.

RESULTS

The Tables 1 and 2 show the number of subjects, age, risk factors, APL and LA titer for the two groups A and B, respectively. Forty patients from group A (80%) had suffered more than one MI episode. 6 of Group A were presented with unstable angina and silent MI. The remaining 4 suffered from severe chest pain. In the control group B, ACA was within the average levels ≤ 10 GPL units/L (Table 2).

In Table 2, smoking was the commonest risk factor in the Group (80%), followed by hyperlipedemia 20% and positive family history (16%). APLs were detected in 44 patients > 12 GPL units/L, which is considered positive (88%) for ACA. 16 patients were positive for LA (32%). The peak value for ACA was 32 GPL units/L for 10 patients (20%). Also, the high level of isotope was IgG in all the patients with only six patients as an exception. It is important to note that there was no patient who suffered from thrombocytopenia. The study has also shown that APLs had no connection with age, frequency of occurrence or the sheer intensity of AP and MI. On the whole, the availability of ACA and L A indicate considerable relationship to the occurrence of MI and AP (P = 0.04), although one is bound to get a different result when we examine them separately.

DISCUSSION

We need to know that ACA and LA are two distinct entities. ACAs had apparent linkage to lamellar Phospho-lipids in a bilayer composition, LAs on the other hand show potent tendency to hexagonal phospholipids, and it is quite normal to identify LAs utilizing lupus sensitive activated partial thromboplastin time (APTT) The cut-off levels of IgG and IgM for ACA can be found in the guidelines stipulated by the American Association of Clinical Pathologists. It defines negative result as <5GPL units. More than or equal 12GPL are stated as low-positive results; 15-18GPL units are classified as medium level. 80 > GPL units are grouped as high levels (Miyakis et al 2006) and (Lockshin 2008). In another study Low ACA levels were reported on subjects with frequent miscarriage (Girardi et al 2004)

In the present study, 30 patients had ACA levels 20 to 32 GPL units/L and the peak value was 32 GPL units/L (Table 1). The results has tailed with our hypothesis that

Table 1. Profile of the patients group (Group A).

No of Subjects	Age (years)	Smoking	Hyperlipedemia	FH	Low protein C	Obesity	LA	APL titer (GPL units/L)
Group 1 (n=4)	20-24	+	-	+	-	-	-	ACA 12
Group 2 (n=4)	24- 28	+	-	+	-	-	-	ACA 20
Group 3 (n=16)	28- 32	+	-	-	+	-	-	ACA 23
Group 4 (n=6)	32-36	+	-	-	+	-	+	ACA < 10
Group 5 (n=10)	36-40	+	-	-	-	+	+	ACA 32
Group 6 (n=10)	40-44	-	+	-	-	-	-	ACA 12

ACA = anticardiolipin antibodies in GPL unit/L, LA = lupus anticoagulants, FH = positive family history.

Table 2. The control Group B.

No of subjects	Age (years)	Risk factors	APL titer (GPL units/L)
2	20-24	Smoking + obesity	10
10	24-28	Hyperlipedemia	5 <
8	28-32	---	5 <
14	32-34	Smoking	5 <
4	34-38	Family history	5 <
2	38-42	Obesity	5 <

ACA = anticardiolipin antibodies in GPL unit/L, LA = lupus anticoagulants, FH = positive family history.

the patients in KSA have moderate level of ACA compared to the western countries (Nomura et al., 2003; Lockshin, 2006). Low ACA levels were reported in another study on subjects with frequent abortions (Girardi et al., 2004). The current study has been carried out on patients less than 45 years of age, given the fact that they are less prone to atherosclerosis. A previous study by Graham (2009) had reported 60 to 90% concordance for ACA and LA; however in the present study, 16 cases (32%) showed presence of both ACA and LA (Table 1). We therefore are of the view that samples must be tested for both ACA and LA. Elevated ACAs were present in 20 cases

representing 40%, with the range of 20 to 23 GPL units/L and 10 cases representing (20%) with ACA 32 GPL Units/L (Table 1). These results were in accordance with the study that showed ACAs elevation in 22% of survivors of MI, aged less than 50 years (Graham, 2009).

The high level-positive results > 80 GPL units/L as described in different studies had not been found in the present study. The high peak value of ACA was 32 GPL units/L. The high level-positive figure of ACA seemed to be seen in older patients with atherosclerosis and rheumatic disorders (Asherson et al., 2001). A prospective study revealed that raised levels of ACA at 40 years of

age correlated positively with the incidence of AP and MI, and mortality related to MI 10 to 20 years later (Heilmann et al., 2008). More than 20% of young (< 45 years) survivors of acute MI carry ACA and in those surviving, 61% of the patients with persistent antibodies experienced later another thromboembolic episode (Carl et al., 2008).

A limitation of this study was its retrospective orientation and small number of subjects (50) enrolled. Extensive studies are required on patients with AP and MI in Suadi Arabia. There seemed to be no clear linkage between ACA and MI in patients within these age groups.

The role of LA in arterial thrombosis is generally acknowledged, however the part played by ACA is still debatable (Pierangeli et al., 2000). This is in agreement with our observation that the prevalence of both LA and ACA assumed statistical significance ($p \leq 0.05$) only when considered together and not in disparate form.

Conclusion

This study was conducted on AP and MI Saudi patients below 45 years. The patients group includes 50 patients (Table 1) and 40 normal controls subjects (Table 2) without any other associated disease. APLs had been elevated in 88% of patients, although that did not coincide with the constant recurrence of MI. The high level of APLs did not also relate with the MI intensity. A clear detection of APLs could apparently, give a lead in explaining the patients' risk of having arterial and venous thrombosis. It could also assist in guiding the therapeutic administration of AP and MI patients. Further research on the prevalence of APLs among the Saudi populace is indeed, needed. The study, if carried out with considerable number of controls and patients, would show the role of APLs in the pathogenesis of MI.

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Full Length Research Paper

Detection and typing of human papillomavirus (HPV) in condyloma acuminatum and bowenoid papulosis HybriBio HPV GenoArray test kit, real-time polymerase chain reaction (PCR) and sequencing

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A new method using HybriBio HPV GenoArray test kit was developed for detection of human papillomavirus (HPV) in anogenital non-tumor diseases, which was compared with the real-time PCR, and sequencing for specificity and sensitivity. All initial validation studies with the control DNA proved to be type-specific. Tissue specimens were obtained from 66 patients with external genital condylomata acuminate (n = 28) and bowenoid papulosis (n = 28) and 10 negative controls. HPV 6 DNA and HPV 11 DNA were detected in 14 and 9 of 28 condylomata acuminate patients, respectively and simultaneous infection of HPV 6 and HPV 11 DNA, HPV 11 and HPV 18 DNA were detected in 3 and 1 of 28 condylomata acuminate patients. HPV 16 DNA and HPV 6 DNA were detected in 17 and 1 of 28 bowenoid papulosis patients, and the other ten bowenoid papulosis patients were detected negative. The sensitivity of HybriBio HPV GenoArray test and real-time polymerase chain reaction (PCR) were 97.8% (45/46) and 95.7% (44/46), and the specificity was 100% (20/20) and 95% (19/20). The overall agreement between HybriBio HPV GenoArray test and sequencing was 98.5% (Cohen's kappa value = 0.96), while real-time PCR and sequencing was 95.5% (Cohen's kappa value = 0.89). HPV genotyping was performed by using the newly commercially introduced HPV GenoArray test kit which makes use of both DNA amplification and HybriBio's proprietary flowthrough hybridization technique to simultaneously identify 21 HPV genotypes, with 3 common subtypes of HPV 53, HPV 66 and cp8304 in Chinese, which can potentially be a valuable tool for the detection of HPV DNA.

Key words: Human papillomavirus, condylomata acuminate, bowenoid papulosis.

INTRODUCTION

Human papillomavirus (HPV) is a small DNA virus belonging to the family Papovaviridae which infect squamous cell mostly. More than 100 types of HPV have been identified (Syrjanen et al., 2003). The HPV infection of the vulva and anogenital region is reflected in a spectrum of histological changes. Condylomatous verrucous lesions, smaller papular or plaque-like changes with vulvar intraepithelial neoplasms (VIN) histological features, as well as infiltrative malignant neoplasia with certain histological properties, could be induced by different HPV types. A typical morphologically well-differentiated change is condyloma acuminatum, with the pathological finding of acanthosis, hyperkeratosis, parakeratosis, dyskeratosis and koilocytosis (Boon et al., 2005).

Bowenoid papulosis, the lesions which consists of multiple red or brown papules in the genitoanal region, histologically, show alterations of the epidermis similar to those in Bowen's disease. The course of the disease is benign; however, malignant transformation has been documented (Palefsky and Joel, 2007). Female sexual partners of men with this disease may be at increased risk of cervical cancer (Gesierich et al., 2008). The aetiopathogenesis of the disorder is not well defined but it

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may be linked to HPV infection. Therefore, clinical diagnosis may not be sufficient and histopathological diagnosis and virological tests are necessary. Moreover, since high-risk types of HPV associated with cervical cancer and Bowenoid papulosis have been detected, identification of genotypes is preferable. The purpose of this study was to confirm diagnosis and to detect the type of HPV associated with vulvar condyloma acuminate and Bowenoid papulosis by three methods of HybriBio HPV GenoArray test, real-time polymerase chain reaction (PCR) and sequencing.

MATERIALS AND METHODS

Clinical samples

Fifty-six patients (31 males and 25 females; mean age, 34 [range, 18 to 75 years]) with external genital polypoid lesions entered the study at Huashan Hospital outpatient clinic between 2008 and 2010. The study was conducted in accordance with the guidelines of the Ethical Review Board of the Fudan University School of Medicine. For each patient, biopsy specimens measuring 3 mm in diameter were taken from two lesions. The specimens were used both for routine histopathological diagnosis and for HPV detection and genotyping.

Diagnostic criteria

The people whose anogenital pathologic diagnosis is condylomata acuminate or Bowenoid papulosis could enter the study.

Exclusion criteria

The patients who have any kinds of treatments or any complications could not enter the study and specimens biopsied from cervix would be excluded.

DNA extraction

DNA was extracted from the specimen using QIAamp DNA Kit (Qiagen, Chatsworth, CA). After extraction, DNA was eluted in 200 μl distilled water and was stored at -20°C.

HybriBio HPV genoarray

HPV genotyping was performed by using the HPV GenoArray test kit (Hybribio Limited, Hong Kong), which makes use of both DNA amplification and HybriBio's proprietary flowthrough hybridization technique to simultaneously identify 21 HPV genotypes genotypes, including 5 low-risk types (types 6, 11, 42, 43, and 44), 14 high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and 2 intermediate-risk types (types CP8304 and 53) (Grisaru D et al., 2008). The test employs a macroarray format with a nylon membrane onto which HPV genotype-specific oligonucleotides probes have been immobilized.

DNA extraction

DNA was extracted from the specimen using QIAamp DNA Kit (Qiagen, Chatsworth, CA). After extraction, DNA was eluted in 200 μl distilled water and was stored at -20°C.

Real-time polymerase chain reaction (PCR)

Type-specific real-time PCR was used to measure the quantity of the DNAs of HPV 6, 11, 16, and 18 in each sample. The sequences of primers and probes for E6/E7 region used have been listed below (Table 1). PCR reactions were carried out using the TaqMan PCR Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's directions. Standard curves measuring HPV 6, 11, 16, and 18 DNA concentrations were constructed using cycle threshold (CT) values obtained from serially diluted plasmids, pBR322, respectively, which contain the target DNA sequences. The CT value from each sample was plotted on a standard curve, allowing automatic calculation of the copy number using Sequence Detector v1.6 software (PE Applied Biosystems). Each sample was tested in duplicate; the copy number of each sample is represented as the mean of the two values.

Direct HPV DNA sequencing

All samples were amplified with E6/E7 primers and purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. After purification, 2 μl of each amplicon was tested by electrophoresis on 2% agarose gel for the evaluation of the quality and the quantity of amplified DNA. The purified amplicons were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The reaction products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the resulting sequences were compared with HPV sequences of known types using the basic local alignment search tool from the NCBI website (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Statistical analysis

The degree of agreement between HybriBio HPV GenoArray test, real-time PCR and sequencing in detecting HPV infection was calculated by Cohen's kappa values. Kappa values of less than 0, 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8, 0.81 to 0.99 and 1.0 designate no, slight, fair, moderate, substantial, almost perfect and perfect agreements, respectively.

RESULTS

The results detected by HybriBio HPV GenoArray test

HPV 6 and 11 DNA were detected in 14(50%) and 9(32.1%) of 28 condylomata acuminata, respectively, and simultaneous infection of HPV 6/11 DNA, HPV 11/18 DNA were detected in 3(10.7%) and 1(3.6%) of 28 condylomata acuminate, 1 (3.6%) detected negative. HPV 6 DNA and HPV 16 DNA were detected in 1 (3.5%) and 17 (60.7%) of 28 Bowenoid papulosis. 10 (35.7%) Bowenoid papulosis were detected negative. The controls were detected negative (Table 2 and Figure 1).

The results detected by real-time PCR

HPV 6/11 DNA were detected in 26 (92.9%) of 28 condylomata acuminata, respectively, and 2 (7.1%) specimens were detected negative. HPV 16 DNA, HPV 6/11 DNA and HPV 18 DNA were detected in 17 (60.7%),

Table 1. Primers and probes used for real-time PCR detection of HPV types.

HPV type	Primer and probe sequences	Target gene
6	F: 5'-CGGTTGATAAAGCTAAATTGTACGT-3' R: 5'-AGGGTAACATGTCTTCCATGCA-3' P: 5'-AAGGGTCGCTGCCTACACTGCTGG-3'	E6
11	F: 5'-GCTTCATAAAACTAAATAACCAGTGGAA-3' R: 5'-GTCAGGAGGCTGCAGGTCTAGTA-3' P: 5'-CTATATCCTTTAGGGTAACAAGTCTTCCATGCATGTTG-3'	E6
16	F: 5'-TTGCAGATCATCAAGAACACGTAGA-3' R: 5'-CAGTAGAGATCAGTTGTCTCTGGTTGC-3' P: 5'-AATCATGCATGGAGATACACCTACATTGCATGA-3'	E7
18	F: 5'-AGAGGCCAGTGCCATTCGT-3' R: 5'-GTTTCTCTGCGTCGTTGGAGT-3' P: 5'-TCCTGTCGTGCTCGGTTGCAGC-3'	E6
Control	F: 5'-CTGGCACCCAGCACAATG-3' R: 5'-GCCGATCCACACGGAGTACG-3' P: 5'-ATCAAGATCATTGCTCCTCCTGAGCGC-3'	beta-actin

F, forward primer; R, reverse primer; P, probe.

Table 2. The results detected by HybriBio HPV GenoArray test.

Specimen	HPV 6	HPV 11	HPV 16	HPV 18	HPV 6, 11	HPV 11, 18	Negative
CA	14	9	0	0	3	1	1
BP	1	0	17	0	0	0	10
Controls	0	0	0	0	0	0	10

Table 3. The results detected by real-time PCR.

Specimens	HPV 6/11	HPV 16	HPV 18	Negative
CA	26	0	0	2
BP	1	17	1	9
Controls	0	0	0	10

1 (3.6%) and 1 (3.6%) of 28 Bowenoid papulosis, respectively. 9 (32.1%) Bowenoid papulosis were detected negative. The controls were all detected negative (Table 3 and Figure 2).

The results detected by sequencing

All specimens were detected by sequencing as the gold standard, with the same results by HybriBio HPV GenoArray test and real-time PCR, except for the five, in which one had pathologic diagnosis to be bowenoid papulosis and the other four were condyloma acuminatum (Table 4).

Sensitivity and specificity and the overall agreement between sequencing and HybriBio HPV GenoArray test or real-time PCR

The sensitivity of HybriBio HPV GenoArray test and real-time PCR were 97.8% (45/46) and 95.7% (44/46), and the specificity was 100% (20/20) and 95% (19/20), regarding sequencing as golden standard. The overall agreement between HybriBio HPV GenoArray test and sequencing was 98.5% (Cohen's kappa value = 0.96), while the agreement between real-time PCR and sequencing was 95.5% (Cohen's kappa value = 0.89).

DISCUSSION

HPV pertain to the Papillomaviridae family, a highly diverse group of viruses that infect the skin and mucosal epithelia of several vertebrate species. Forty HPV types

Table 4. The five cases with the different results detected by the three methods.

Pathologic diagnosis	HybriBio	Real-time PCR	Sequencing
BP	Negative	HPV18	Negative
CA	HPV6	Negative	HPV6
CA	HPV11	Negative	HPV11
CA	Negative	HPV6/11	HPV6
CA	HPV11, HPV18	HPV6/11	HPV11, HPV18
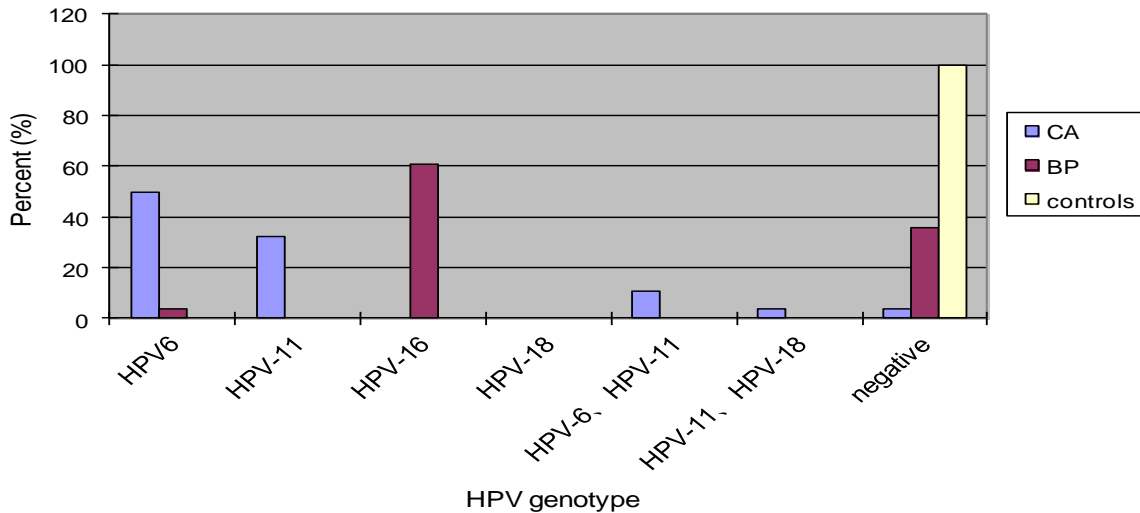
Figure 1. The percents of each HPV genotype detected by HybriBio HPV GenoArray test in the specimens of CA and BP.
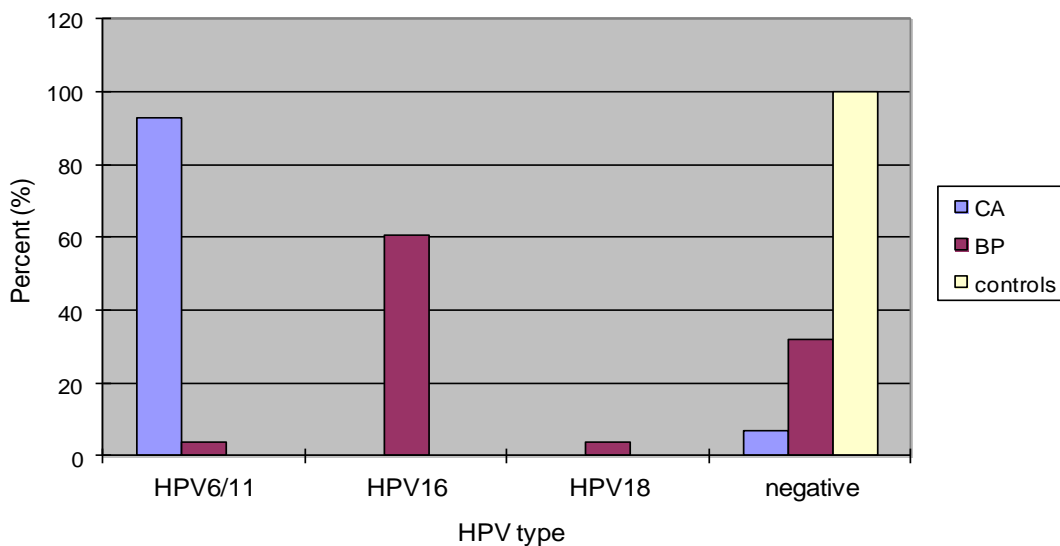
Figure 2. The percents of each HPV genotype detected by real-time PCR in the specimens of CA and BP

belonging to the a-papillomavirus genus have been detected infecting the epithelium and mucosa lining the anogenital tract (Boccardo et al., 2010). HPV types have been further classified based on their oncogenic potential as low- or high-risk HPV types. Low-risk HPV types (that is, HPV 6 and HPV 11) cause common genital warts,

such as condyloma acuminatum, benign hyperproliferative lesions with limited tendency to malignant progression. In our test, 92.8% (26/28) condylomata acuminate were detected HPV 6 or 11 or both, and 3.6% (1/28) was detected HPV 11/18 DNA simultaneous infection by both HybriBio HPV GenoArray Test and sequencing.

Several HPV genotypes are detected in the infection of Bowenoid papulosis, including HPV 16, 18, 31, 35, 39, 42, 48 (Gross and Pfister, 2004), especially HPV 16 infection, which is strongly associated with the external genital squamous cell carcinoma *in situ* such as Bowen's disease and Queyrat erythroplasia (Naoto et al., 2006). The results that 60.7% Bowenoid papulosis infected by HPV 16 detected by the means of both HybriBio HPV GenoArray test and real-time PCR consist in the published data (Naoto et al., 2006), while 3.5% (1/28) was detected HPV 6 corresponding to the spontaneous remission of BP.

HPV detection is important to identify patients who may be at high risk of developing squamous cell carcinoma, though it is clinically difficult to determine whether it is necessary to treat HPV associated anogenital tumors. Virological testing includes *in situ* hybridization (ISH), Southern blot hybridization method, dot blot hybridization, PCR, and real-time PCR. However, detection power using ISH is low. In addition, PCR and real-time PCR need specific expensive equipment such as a thermal cycler, and these methods have not yet become common procedures in hospital laboratories (Masanori et al., 2007). Now we used the HybriBio GenoArray test for HPV genotyping, this assay is a commercial kit which was recently introduced into the market. Although it has already been used for some time in China, some literatures are available to support its value in the high-risk HPV detection (Wang et al., 2012; Liu et al., 2010; Dan et al., 2008). There is little study about its use in condyloma acuminatum and Bowenoid papulosis. Nevertheless, the results of this study show a high level (98.5%) of agreement between the results of that assay and those of sequencing, while the agreement between real-time PCR and sequencing is 95.5%, but the two Cohen's kappas show "almost perfect" agreement between them. This high level of concordance is encouraging, since regarding sequencing as the golden standards and real-time PCR had gained a good reputation over the past few years as a sensitive, specific, and reliable test for the detection of infections with high risk and low risk (Guo et al., 2002) HPV types.

Conclusion

This study indicates that the HybriBio HPV GenoArray test is shown as reliable for clinical performance as does the real-time PCR test. The HybriBio HPV GenoArray method is superior in terms of sensitivity, specificity, speed, and simplicity, and can be a valuable tool for the detection of HPV DNA in laboratories.

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Full Length Research Paper

Retrospective analysis of medication for 508 cases of type II diabetes patients

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The aim of this study was to investigate the medication status of 508 patients with type II diabetes admitted to Huashan Hospital, and to guide their clinical medication. The data of the 508 patients with type II diabetes who were admitted to Huashan Hospital between July, 2010 and December, 2010 were collected and analyzed. In 508 patients, acarbose was the most frequently prescribed medication (n = 337, 66.34%), the highest frequency of acarbose was 66.34% (n = 337). Metformin which belongs to the biguanide group accounted for 20.28%. Pioglitazone which belongs to thiazolidinedione group had the lowest frequency of use, and was only 1.38% (n = 7), and patients were often treated with combination therapy (n = 194, 38.18 %). A total of 431 patients (85%) had chronic diabetes complications (state which ones: macro or micro vascular complications). Among the diabetic patients, age groups in 40 to 80 had the highest probability of illness and the incidence rate increases with age; therefore, this group should take regular medical examinations to prevent diabetes induced complications. In the choice of medication, prescription drugs like acarbose and other α-glucosidase inhibitors, at the same time, combined medication with pancreatic kinionogenase are preferred. Enteric-coated tablets or other drugs to treat high blood pressure diabetes, heart disease, nervous system damage and other complications are also recommended.

Key words: Type II diabetes, combined medication, age related, complication, biguanide.

INTRODUCTION

Diabetes is a chronic metabolic disorder induced by multiple pathogenic factors, if blood glucose is not well controlled, complications will occur, resulting in the lesions of failure of kidney, eye, feet and other parts which cannot be cured. Type II diabetes prevalence in recent years have significantly increased with the increased proportion of obese or overweight. Some studies had shown that metformin has a good effect on normal weight. Therefore, metforminon was preferred on the basis of the lifestyle intervention, if you cannot meet the standard, then take further measures. Taking into account the weight loss, gastrointestinal reactions, and other factors among some patients, if they are not suitable for using metformin, the other drugs should be taken into consideration (Chinese Diabetes Society,

2010). At present, the number of diabetes patients is rapidly rising around the world, which has impacted people's health and quality of life; it has also been ranked as the non-communicable disease second only to cardiovascular diseases and malignant tumors (Hui and Zhi-you, 2008) in occurrence. Diabetes is clinically divided into two types; type I diabetes and type II diabetes which accounts for 95% in china (Heng-jie and Hong, 2008). In China, areas with higher living standard have higher incidence of diabetes. In clinical practice, patients with type II diabetes are usually treated with oral administration of hypoglycemic agents. Hypoglycemic agents with different modes of action have successively gone on the market, which have provided more choices for the treatment of diabetes (Krentz and Bailey, 2005). To investigate the clinical application status of such drugs, data of 508 cases of patients with type II diabetes in our hospital were collected and retrospectively analyzed. Diabetic patients complicated with vascular

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diseases such as early diabetic nephropathy, hypertension and cerebral thrombosis should also use pancreatic kinionogenase enteric-coated tablets, which has effects such as dilation of blood vessels, promotion of microcirculation, activation of plasminogen, reduction of blood viscosity, inhibition of phospholipase A2, prevention of platelet aggregation, daily application of less than 100 mg aspirin would contribute to the prevention of cardiovascular and cerebrovascular diseases. Calcium antagonists and angiotensin-converting enzyme inhibitors are beneficial in diabetes mellitus complicated with hypertension, both types of drugs can reduce left ventricular hypertrophy and enable short-term reduction of proteinuria, but a conclusive result is still difficult to be obtained from long-term follow-up (Hao-lin and Nan-sen, 1999). Several studies have shown that angiotensin-converting enzyme inhibitors can not only lower blood pressure, but also reduce blood glucose and improve glucose tolerance (Baron et al., 1993).

MATERIALS AND METHODS

Data on cases of patients with type II diabetes who were admitted to Huashan Hospital between July, 2010 and December, 2010 were collected and statistical analysis on age, gender, medication status, and diabetic complications were conducted.

Patients

In this study we enrolled 83 patients (43 women and 40 men) with type II diabetes (Nephropathy), 84 patients (52 women and 32 men) with peripheral neuropathy, 88 patients (54 women and 34 men) with retinopathy, 81patients (46 women and 35 men) with coronary heart disease, 81 patients (39 women and 42 men) with cerebral vascular disease, and 20 healthy control subjects (47 women and 44 men). In Table 1, the clinical characteristics of all patients were reported. The microvascular and macrovascular complications only were analysed. The complications were evaluated by four biochemical indices and the whole group was classified by age and gender.

We had obtained ethics approval from the ethics committee at our institution and obtained written informed consent from all involved participants.

Biological indication test

Blood samples of all patients were collected from a peripheral vein and then kept on ice. The serum was collected by centrifugation (3,000 rpm for 10 min at 4°C), aliquoted, and stored at -80°C until analyzed. HbA1c, total cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were examined, respectively. Each experiment was performed in duplicate using same procedure.

RESULTS

Biochemical characteristics of 431 patients who have complications are shown in Table 1. The patients' age ranging from 60 to 80 and men were found to be more likely to get more complications among the type II diabetes patients. All of the patients had higher HbA1c level than normal (<7%). The total cholesterol level also exceeded the normal level. As for AST and ALT level, cerebral vascular disease and peripheral vascular disease had high level of AST, and three complications among peripheral neuropathy, retinopathy and cerebral vascular disease had high ALT level. In short, all the parameters examined by us were higher than the control range.

Complications of diabetes

In 508 cases of patients with type II diabetes, 431 cases had complications such as nephropathy, peripheral neuropathy, retinopathy, coronary heart disease, cerebral vascular disease, peripheral vascular disease, etc., which were accounting for 84.84%; only 77 cases were free of complications. This area needs to be expanded as we believe it is an important rationale for the study.

Medication status of 508 cases of patients with type II diabetes

A total of eight different hypoglycemic agents were used, drug varieties and frequency of use are shown in Table 2. It can be seen from Table 2 that the highest frequency of use was of acarbose at 66.34% (n = 337), metformin which belonged to the biguanide, accounted for 20.28%. Pioglitazone which belonged to thiazolidinedione group had the lowest frequency of use, and was only 1.38% (n = 7); among 508 cases of patients with type II diabetes, 212 cases were administered with only one kind of hypoglycemic agent, 194 cases were administered with two kinds of hypoglycemic agents, and 34 cases with three kinds of hypoglycemic agents.

Concomitant use of hypoglycemic agents with other drugs

Among 508 participants, in addition to the combined use of hypoglycemic agents, other types of drugs were concomitantly used (n = 426 cases, 83.86 %). Concomitantly used drug varieties, frequency of occurrence, and frequency of use are shown in Table 3.

It can be seen from Table 3 that combined medication with pancreatic kinionogenase enteric-coated tablet or other drugs to treat high blood pressure diabetes, heart disease, nervous system damage and other complications was recommended, other combined drugs like aspirin enteric-coated, atorvastatin calcium and irbesartan tablets also had the superiority to other drugs in treating diabetes disease.

Table 1. Biochemical characteristics of 431 patients who have complications.

Parameter	Microvascular complications			Macrovascular complications			Control range
	Nephropathy	Peripheral neuropathy	Retinopathy	Coronary Heart disease	Cerebral vascular disease	Peripheral vascular disease	
Age	62.3	61.5	65.2	80.1	68.3	69.5	60.00
Gender	35M/46F	41M/28F	43M/30F	35M/31F	30M/36F	36M/40F	40M/31F
HbA1c	8.1%	7.9%	7.6%	8.3%	8.5%	8.2%	6.5~7%
Total cholesterol	180±1.2	192±2.5	200±3.6	176±2.4	152±3.8	134±4.4	130~200
AST	56±3	61±4	73±5	68±2	110.2	105	5~40
ALT	42±3	112	109	48	113	76	7~56

Table 2. Type and use proportion of oral hypoglycemic agents

Drug category	Drug name	Number of cases	Frequency of use (%)
α-glucosidase inhibitors	Acarbose (Glucobay)	337	66.34
Biguanides	Metfomin (Junlida)	103	20.28
Sulfonylureas	Glimepiride (Amaryl)	45	8.86
	Gliquidone	17	3.35
	Glipizide (Mieteni)	11	2.17
Thiazolidinediones	Pioglitazone (Aiting)	7	1.38

DISCUSSION

The initial management of type II diabetes involves modifications to diet and exercise therapy; however, if blood glucose levels are not within normal limits then pharmacotherapy is required. If pharmacotherapy is not used when blood glucose levels are consistently high then, the risk for complications will be very high. Acarbose is the most frequently used hypoglycemic agent, its mechanism of action is to inhibit the activity of intestinal-type cells, α-glucosidase, and to delay the hydrolysis of carbohydrates, production of glucose, as well as the absorption of glucose. As a first-line drug for type II diabetes, it is especially suitable for those with normal (or not too high) fasting blood glucose and significantly increased postprandial blood glucose to use alone or in combination with other hypoglycemic agents; adverse reactions are common gastrointestinal reactions. In individual cases, allergic skin reactions such as erythema, rash and urticarial eruption may also occur.

Metformin can improve insulin sensitivity, in addition to hypoglycemic effect, it also has many other effects: it can reduce very low density lipoprotein level, increase high density lipoprotein level, increase arterial blood flow, inhibit platelet aggregation, reduce vascular permeability, inhibit advanced glycation end products, etc (Ofenstein et al., 1999; Ruggierol et al., 1999; Lannello et al., 2004; Heng-zhong et al., 2007). A study has shown that oral administration of metformin can reduce the level of circulating free fatty acids. Some patients showed gastrointestinal discomfort after oral administration of this drug, such as nausea, vomiting, diarrhea, abdominal pain, constipation, abdominal distension and indigestion; type II diabetic patients who were complicated with ketoacidosis, liver and kidney dysfunction, heart failure, acute myocardial infarction, etc., should not use this drug.

For patients with normal renal function, plasma drug tl/2=2-5 h, 90% of absorbed drug was eliminated within 12 h (Bailey and Turner, 1996). The application of sulfonylurea hypoglycemic agents, especially in the elderly, is not recommended, as long-term application could

Table 3. Concomitantly used drug types and number of cases

Drug name	Number of cases	Frequency of use (%)
Pancreatic Kininogenase Enteric-coated Tablets	416	81.89
Aspirin Enteric-coated Tablets	163	32.09
Atorvastatin Calcium Tablets	150	29.53
Irbesartan Tablets	101	19.88
Nifedipine Controlled-Release Tablets	71	13.98
Fenofibrate Capsules	52	10.24
Isosorbide Mononitrate Sustained-Release Tablets	46	9.06
Bisoprolol Fumarate Tablets	48	9.45
Enalapril Maleate Tablets	53	10.43
Nifedipine Tablets	69	13.58
Valsartan Capsules	35	6.89
Metoprolol Tartrate Tablets	21	4.13

lead to high risk of hypoglycemia; for diabetic patients complicated with renal diseases, the appropriate oral hypoglycemic agent is gliquidone, as only 5% of the drug is excreted via the kidney. It is a well known fact that pancreatic kinionogenase enteric-coated tablet combined with epalrestat can have efficiency on diabetic peripheral neuropathy (DPN) patients (Xiao-yuan, 2011; Berkow, 1992; Gulixian et al., 2010; Hui et al., 2008; Ming et al., 2011; Xin-lian and You-yu, 2003). In the combined use of two kinds of drugs, the combination of α-glucosidase inhibitor and insulin was the most prevalent. In the combined use of three kinds of drugs, the combination of α-glucosidase inhibitor, metformin and insulin was the most common. Among the diabetic patients, age groups in 40 to 80 had the higher probability of illness and the incidence rate tend to increase with age; therefore, this group should take regular medical examinations to prevent diabetes regularly. In the choice of medication, prescription drugs like acarbose and other α-glucosidase inhibitors, at the same time, combined medication with pancreatic kinionogenase enteric-coated tablet or other drugs to treat high blood pressure diabetes, heart disease, nervous system damage and other complications is recommended. During the nursing period, blood pressure was controlled below 125/75 mmHg (1 mmHg = 0.133 kPa), and have close observation of changes in blood pressure and prevent the occurrence of orthostatic hypotension. Lower protein in urine would occur by eating the low protein food, thereby reducing the ability of insulin resistance, thus improving glycometabolism, fat metabolism, and protein metabolism, and at the same time, improving injuries, pressure ulcers, and infection prevention. Arteriovenous fistula should be taken care for dialysis patients (Ji-qiong et al., 2011).

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Full Length Research Paper

Relaxation versus diffusion on the diclofenac sodium release from matrix tablets containing hydroxypropylmethylcellulose and/or chitosan

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Different formulations of diclofenac sodium (DS) containing hydroxypropylmethylcellulose (HPMC) and/or chitosan were prepared, with a view to appraise the effect of the said polymers on the drug release behaviour from matrix tablets prepared by the direct compression method. The tablets were tested for different assays, including swelling and release performance. Differential scanning calorimetry (DSC) and Raman spectroscopy were performed in order to estimate the compatibility between the matrix components (DS and excipients). From the DSC and Raman results, non-negligible drug:excipient interactions were detected, although, these were found not to constitute an incompatibility effect. The dissolution tests and the kinetic analysis data indicated that the rate and the mechanism of DS release from tablets are mainly controlled by the drug/polymer ratio. The release rate became slower for a high polymer content of HPMC. Moreover, the results demonstrated that chitosan could accelerate the drug release with lower amount in the formulation. The analysis of the drug release profile was performed in the light of distinct kinetic mathematical models. Release from formulations F2 and F3 occurs by an anomalous transport mechanism (coupling of diffusion/erosion mechanisms), with Kosmeyer-Peppas exponent (n) values of 0.626 and 0.706, respectively. The balance between diffusion and polymer erosion competing mechanisms of drug release were assessed by the Peppas-Sahlin model.

Key words: Diclofenac sodium (DS), drug release, hydroxypropylmethylcellulose (HPMC), chitosan, Raman spectroscopy, differential scanning calorimetry (DSC).

INTRODUCTION

Diclofenac sodium (DS) [2-[(2,6-Dichlorophenyl)amino] benzeneacetic acid monosodium salt] is a synthetic non-steroidal anti-inflammatory drug (NSAID) that has anti-inflammatory, analgesic, and antipyretic properties. It is used for the treatment of degenerative joint diseases such as rheumatoid arthritis, osteoarthritis, and ankylosing spondilitis (Adeyeye and Li, 1990).

DS is rapidly dissolved in intestinal fluid reaching its maximum blood concentration (C_{max}) within 30 min and is metabolised mainly by hepatic hydroxylation and subsequent conjugation. In healthy human volunteers, mean elimination half-life of the terminal phase was found to be 1.2 to 1.8 h (Fowler et al., 1983). Due to its rapid elimination, a controlled release dosage form, allowing the maintenance of the DS therapeutic level for a longer

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time period, improving the pharmacological activity, and reducing toxic effects would be very appreciated by the patients (Bravo et al., 2002).

Matrix type formulations appear to be a very attractive approach from both process development and scale up points of view. They consist of a system for delaying and controlling the release of a drug which is dissolved or dispersed in a support resisting to disintegration. One method to prepare controlled-release formulations is the incorporation of the active principle in a matrix containing a hydrophilic, rate-controlling polymer (Li et al., 2005; Mourão et al., 2010). Recently, studies have shown the effect of polymer blends on release profiles of DS from matrices and the results evidenced a dependence of the drug release on the kind of polymer and also on its proportion in the formulation (Samani et al., 2003; Mourão et al., 2010).

Hydroxypropylmethylcellulose (HPMC) is the most important hydrophilic polymer used for the preparation of oral controlled release drug systems, due to its non-toxic nature, its capacity to incorporate active principles of varying characteristics, its non-pH dependence, its swelling properties which have a considerable effect on the release kinetics of the incorporated drug (Ghimire et al., 2010). Water penetration, polymer swelling, drug dissolution, drug diffusion, and matrix erosion from these dosage forms are controlled by the hydration of HPMC, which forms a gel barrier through which the drug is able to diffuse (Velasco et al., 1999; Siepmann et al., 2002; Vueba et al., 2005). The influence of the DS:HPMC ratio, particle size of the drug and the polymer, and the compression force, on the drug release process from HPMC matrices was evaluated by Hiremath and Saha (2008), showing that the rate and the drug release mechanism are mainly controlled by the drug:HPMC ratio. Tablets prepared using HPMC on contact with aqueous fluids gets hydrated to form a viscous gel layer through which drug will be released by diffusion and/or by erosion of the matrix (Katzhendler et al., 2000). Moreover, studies carried out before (Kim and Fassihi, 1997), has demonstrated that the hydration-gelation contributes to the development of swelling/erosion boundaries and consequently to constant drug release. Combination of these of two polymers facilitates rapid formation of necessary boundaries (that is, gel layer and solid core boundaries) to control overall mass transfer processes.

Chitosan [Poly-(1-4)-2-Amino-2-deoxy-β-D-Glucan], in turn, is a linear cationic polysaccharide obtained by N-deacetylation of chitin, a naturally-occurring structural polysaccharide abundant in crab and shrimp shells. It has recently attracted great attention in the pharmaceutical and biomedical fields due to its favourable biological properties such as biocompatibility, inertness, versatility, and biodegradability. The conjugation of chitosan to various medicinal agents is also facilitated by its nature as an amino sugar polymer. Moreover, chitosan has antacid and antiulcer activities, which may prevent or weaken drug-induced irritation in the stomach. All these interesting properties render this natural polymer an ideal candidate for controlled drug release formulations (Majeti and Ravi, 2000; George and Abraham, 2006).

The aim of this study is to evaluate the effect of both a cellulose ether polymer, HPMC K15M, and a non-cellulose semi-synthetic polymer, chitosan, on the release behaviour of the DS active principle from a matrix tablet system, using distinct formulations in order to understand how they rule this process.

MATERIALS AND METHODS

Drug: DS (BP grade), Capsifar, Oeiras, Portugal. Polymers: hydroxypropylmethylcellulose, Methocel® (HPMC K15M), England and chitosan (90.5% deacetylation degree), Exquim S.A, Barcelona, Spain. Diluent: lactose monohydrate (LAC), Granulac® 200, Meggle, Wasserburg, Germany. Magnesium stearate was used as lubricant (Analytical grade).

Pre-formulation studies

Differential scanning calorimetry (DSC)

DSC measurements were performed using a Shimadzu DSC-50 with a thermal analyser (Shimadzu TA-50, Tokyo, Japan). About 3 mg of either drug or excipient, or 6 mg of the drug/excipient 1:1 (w/w) mixture were analysed, in sealed aluminium pans under nitrogen flow (20 ml/min), at a heating rate of 10°C min^{-1}, from 25 to 350°C. An empty sealed pan was used as reference. The equipment was calibrated with indium (99.98%, m.p.156.65°C, Aldrich®, Milwaukee, USA).

Raman spectroscopy

The Raman spectra were obtained on a triple monochromator Jobin-Yvon T64000 Raman system (focal distance, 0.640 m; aperture, f/7.5) equipped with holographic gratings of 1800 grooves/mm. The premonochromator stage was used in the subtractive mode. The detection system was a liquid nitrogen cooled non-intensified 1024 × 256 pixel (1") Charge Coupled Device (CCD). A Coherent (model Innova 300-05) Ar$^+$ laser was used as the light source, the output of which, at 514.5 nm, was adjusted to provide 50 mW at the sample position. A 90° geometry between the incident radiation and the collecting system was employed. The entrance slit was set to 100 μm. 5 scans with integration times of 60 s for pure DS and DS:HPMC K15M mixture and 3 s for DS:LAC mixture, were used in all the experiments. Samples were sealed in Kimax glass capillary tubes of 0.8 mm inner diameter. Under the aforementioned conditions, the error in wavenumbers was estimated to be within 1 cm^{-1}.

Preparation of the matrix tablets

The distinct formulations of the matrix tablets analysed in this study are provided in Table 1. Matrix tablets were produced by varying both the polymer and diluent content, for a fixed amount of drug, 100 mg. DS, polymer or polymer mixture, and diluent were passed through a 100 mesh sieve and were thoroughly mixed in a plastic bag for 15 min. Magnesium stearate (lubricant) was also sieved

(500 mesh), added to the previous mixture, and blended for an extra 5 min. All matrices (total mass of 276 mg) were prepared by direct compression in an automatic hydraulic press (Specac Press, England), using flat 10 mm diameter punches and a compaction pressure of 624 MPa, as described by Vueba et al. (2004).

DS quantification in the matrix tablets

Five randomly chosen tablets of each formulation were thinly minced in a mortar. 41.4 mg of the resulting powder was solubilised in phosphate buffer (pH 6.8), up to a final volume of 500 ml. Several aliquots were then filtered and assayed by UV spectrometry at 275 nm (Shimadzu UV-1603 spectrometer). The determination was carried out as described in USP 34 (2011), the results reported being the average of 3 independent measurements.

Characterization of the tablets

Mass uniformity of the tablets

A total of 30 tablets of each formulation were evaluated for their weight, using an analytical balance (KERN 770). The results were expressed as mean values of 30 independent determinations, according to USP 34 (2011).

Tablet thickness

The thickness of the matrix tablets was determined using a micrometer (Roche, Switzerland) and the results were expressed as mean values of 10 individual tablets of each formulation.

Hardness determination

The hardness of the tablets was determined using a tablet hardness tester (Erweka TBH28, Erweka GmbH, Germany) and the results were expressed as the mean of 10 determinations.

Mechanical tensile strength

The tensile strength (T) of the tablets was assessed on a tablet hardness tester (Erweka TBH28, Erweka GmbH, Germany), for 10 tablets of each formulation, from the force required to fracture them by diametral compression, according to the following equation:

$$T = \frac{2P}{\pi Dt} \tag{1}$$

where P represents the applied load, and D and t are the diameter and thickness of the tablet, respectively (Fell and Newton, 1970).

Friability

Twenty tablets were weighed and placed into a friability tester (Erweka TA20, Erweka GmbH, Germany). The tablets were subject to 25 rpm for 4 min and were then re-weighted to obtain the friability, by determining the weight before and after the test. This process was repeated for all formulations and the percentage friability was calculated using the following equation:

$$F = \frac{W_1 - W_2}{W_1} \times 100 \tag{2}$$

where F represents the percentage weight loss, and W_1 and W_2 are the initial and final tablets weights, respectively.

Swelling studies

Swelling studies were carried out for all formulations tested. Three metallic baskets were weighed with a tablet from each formulation and were placed in 1000 ml of phosphate buffer pH = 6.8 at 37.0 ± 0.5°C. At hourly intervals, the baskets were taken out from the vessel, gently wiped with a tissue to remove surface water, re-weighted and placed back into the vessel as quickly as possible. The mean weights were determined for each formulation, and the swelling degree (S) was calculated according to the relationship (Efentakis et al., 1997):

$$S = \frac{W_s - W_d}{W_d} \times 100 \tag{3}$$

where W_d and W_s are the dry and swollen matrix weights, respectively. The swelling degree was the mean of 3 independent assays.

Drug release analysis

Dissolution studies were performed according to the USP 34 paddle method (2011). The dissolution medium was phosphate buffer (pH = 6.8, 1000 ml) at 37.0 ± 0.5°C, and a stirring speed of 100 rpm was used. Six different tablets were tested in six dissolution vessels (Vankel VK-7000 dissolution testing station, in-line with a closed flow through system using a peristaltic pump, connected to a Shimadzu UV-1603 spectrophotometer). The progress of the dissolution process was monitored by determining the amount of DS spectrometrically, at 275 nm, for samples withdrawn and filtered every 5 min, for a total of 1200 min. The corresponding drug-release profiles were represented by plots of the cumulative percentage of drug release (calculated from the total amount of DS contained in each matrix) versus time.

Kinetic mechanism

Several mathematical models can be used to describe the kinetic behaviour of the drug release mechanism from matrix tablets; the most suited one being that which best fits the experimental results. The choice of a specific model for a particular data set depends on the shape of the plot obtained, as well as on the underlying mechanism. The kinetics of DS release from hydrophilic cellulose matrix tablets was determined by finding the best fit of the dissolution data (amount of drug released versus time) to distinct models: zero-order (Equation 4), first-order (Equation 5), and Higuchi (Equation 6) (Higuchi, 1961, 1963).

$$M_t = M_0 + k_0 t \tag{4}$$

where M_t is the amount of drug released at time t, M_0 is the amount of drug in the solution at t = 0 (usually, $M_0 = 0$), and k_0 is the zero-order release constant.

Table 1. Composition of the distinct formulations of DS.

Component	Formulations (mg)			
	F1	F2	F3	F4
DS	100	100	100	100
HPMC K15M	50	100	85	68
Chitosan	–	–	–	17
LAC	125	75	90	90
Magnesium stearate	1	1	1	1

$$M_t = M_\infty (1 - e^{-k_1 t}) \tag{5}$$

M_∞ being the total amount of drug in the matrix and k_1 the first-order kinetic constant.

$$M_t = k_H t^{1/2} \tag{6}$$

k_H representing the Higuchi rate constant.

Moreover, to better characterise the drug release behaviour for the polymeric systems under study, and particularly to gain some insight on the corresponding mechanism, the Korsmeyer-Peppas (Equation 7) semi-empirical model was applied (Korsmeyer et al., 1983).

$$\frac{M_t}{M_\infty} = k_{KP} t^n \tag{7}$$

M_t/M_∞ representing the fraction of drug released at time t, k a constant comprising the structural and geometric characteristics of the tablet, and n, the release exponent, being a parameter which depends on the release mechanism and is used to characterise it (Peppas, 1985). For cylindrical tablets (Ritger and Peppas, 1987), in particular, $n \leq 0.45$ corresponds to a Fickian diffusion release (case I diffusional), $0.45 < n \leq 0.89$ to an anomalous (non-Fickian) transport, $n = 0.89$ to a zero-order (case II) release kinetics, and $n > 0.89$ to a super case II transport.

The direct fitting of the drug release data to the nonlinear equations mentioned earlier is usually avoided through linear transformation of the data, followed by a linear regression analysis. However, this method may not be mathematically accurate, since it uses transformed values (logarithms) instead of the original data (Lu et al., 1996). Consequently, a direct nonlinear fitting of the experimental results was performed in this work, for each of the mathematical models considered (through minimisation of the sum of the squared residuals). Only the points comprised in the interval $0.1 < M_t/M_\infty < 0.6$ were used.

Additionally, to calculate the relative contribution of diffusional and relaxational mechanisms on the drug release, the Peppas-Sahlin heuristic model was applied (Peppas and Sahlin, 1989):

$$\frac{M_t}{M_\infty} = k_F t^m + k_R t^{2m} \tag{8}$$

The first term on the right-hand side of Equation 8 refers to the Fickian diffusional contribution, while the second term represents the case II erosional contribution. The coefficient m is purely Fickian diffusional exponent, that depends on the geometrical shape of the releasing device through its aspect ratio, which, for the flat-faced, disc-shaped used tablets, was calculated to be 3.8 (diameter/thickness). Thus, according to the figure presented by Peppas and Sahlin (1989), the m value is about 0.447. The percentage of drug release through a Fickian mechanism (F) was calculated by Equation 9, whereas the ratio of relaxational (R) over Fickian mechanism was obtained according to Equation 10 (Peppas and Sahlin, 1989):

$$F = \frac{1}{1 + (k_R/k_F) t^m} \tag{9}$$

$$\frac{R}{F} = \frac{k_R}{k_F} t^m \tag{10}$$

Mean dissolution time

To further characterise the drug release, the mean dissolution time (MDT) was calculated according to the following equation:

$$MDT = \frac{\sum_{j=1}^{n} \hat{t}_j \Delta M_j}{\sum_{j=1}^{n} \Delta M_j} \tag{11}$$

where j is the sample number, n is the number of dissolution sample times, $\hat{t}_j$ is the time at midpoint between t_j and t_{j-1}, and ΔM_j is the additional amount of drug dissolved between t_j and t_{j-1}.

Dissolution efficiency

The dissolution efficiency (DE) is defined as the relationship between the area under the curve (AUC) of dissolved percentage, as a time function, at an observed time and the area of a rectangle corresponding to 100% dissolution at the same time, according to the following equation (Khan and Rhodes, 1972; Khan, 1975):

$$DE = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100 \tag{12}$$

where y is the percentage of drug dissolved at time t.

Statistics

In order to assess statistical significance among the data, one way analysis of variance (ANOVA) was used to test variation in tablets formulations containing different polymer (HPMC K15M and Chitosan) at different % w/w and in the same dissolution media. ANOVA was utilized as well as to test differences in the physical characterization of the matrix tablets. The difference between variants was considered significant for $P < 0.05$, followed by Bonferroni comparison t-test. Statistical analysis was done using
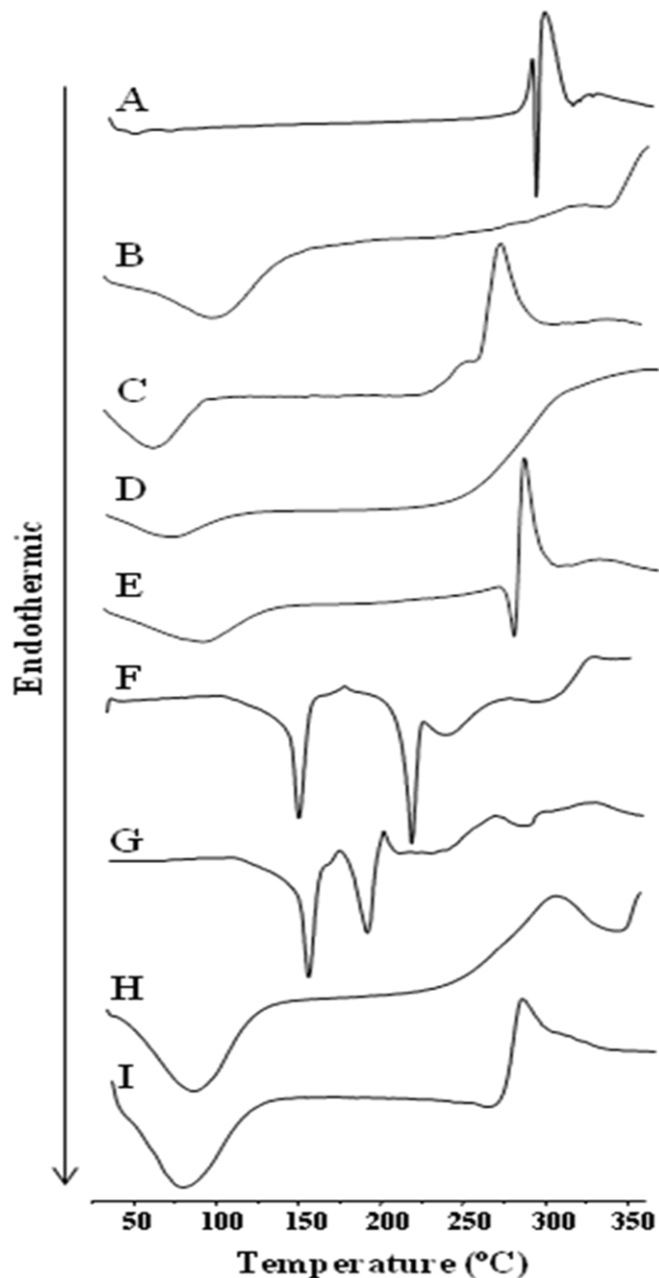
Figure 1. DSC curves of DS (A), HPMC K15M (B), DS:HPMC K15M (C), Chitosan (D), DS:Chitosan (E), LAC (F), DS:LAC (G), Chitosan:HPMC K15M (H), and DS:Chitosan:HPMC K15M (I) in 1:1 (C, E,G, and H) or 1:1:1 (I) (w/w) in the cases of physical mixtures.

Sigma Stat® for Windows (version 2.03, SPSS Inc).

RESULTS AND DISCUSSION

DSC

Pre-formulation studies are an important step in the selection of excipients for formulations of dosage forms.

In fact, some physical or chemical incompatibilities between the drug and the excipients may occur being reflected on: thermal events variation, such as the appearance or disappearance of an endothermic signal; changes in the peak shape and variations in T_{onset} or T_{endset} derived the interactions in the simple mode from DSC curves. In order to investigate the possible interactions between DS and distinct polymers, polymer mixture and/or diluent, DSC was carried out for this purpose (Figure 1). The 1:1 (w/w) ratio was chosen, because it maximises the likelihood of observing any interactions (Mura et al., 1995). The DSC curve of DS was typical of a crystalline anhydrous substance as shown in Figure 1A. The peak temperatures as well as DS enthalpy values are collected in Table 2.

A large broad endothermic effect, over the temperature range 60 to 140°C, was observed for HPMC K15M (Figure 1B), upon evaporation of adsorbed water (Ford, 1999). The DSC curve of chitosan, in turn, was typical of amorphous hydrated compounds, showing a broad endothermic effect ranging between 50 and 100°C (Figure 1D) due to a dehydration process. The exothermic effect observed around 300°C was probably due to the oxidative decomposition or to the glass transition of the sample (Khalid et al., 2002). LAC thermogram, displayed two sharp endothermic peaks, at both 147 and 219°C (Figure 1F). The physical mixture 1:1 (w/w) of both polymers (HPMC K15M/chitosan) did not produce significant modifications on the thermal curve in comparison with that of either HPMC K15M or chitosan (Figure 1H). The combination of the drug with both polymers, DS/HPMC K5M (Figure 1C) and DS/chitosan (Figure 1E), demonstrated an interaction between the components with the drug dispersion in the polymer. A progressive reduction in peak size and a considerable downward shift of the drug peak temperature, causes a decrease of the melting endothermic onset and a reduction of the melting enthalpy (Table 2), suggesting a probable eutectic formation that is actually possible between active drugs and amorphous hydrated polymers (Zalac et al., 1999; Mahendran et al., 2001). The miscibility between the components, in both cases, seems to occur in a large extension.

On the other hand, when LAC was combined with the drug in a 1:1 (w:w) ratio, a significant downward shift of the drug melting peak and also a downward shift of the excipient melting peak were detected, coupled to a broadening effect (Figure 1G). These observations reflect the existence of solid-solid interactions between the two components, demonstrating the mixture of the drug with the diluent in accordance with findings previously reported by other authors (Verma and Garg, 2004; Sipos et al., 2008). Moreover, solid-solid interactions between LAC and ketoprofen were already reported (Batista de Carvalho et al., 2006).

The DSC assay of the drug:HPMC K15M:chitosan 1:1:1 (w/w) mixture (Figure 1I) presented a decrease in

Table 2. Peak temperature and enthalpy values of DS sodium in various drug-polymer mixtures and drug-LAC mixture.

Component	Drug:Excipient (w/w)	T_{peak} (ºC)	T_{onset} (ºC)	T_{endset} (ºC)	ΔH_f corr [a] (J/g)
DS	–	293.72	283.16	313.44	184.77
DS:HPMC K15M	1:1	273.03	262.23	284.70	96.88
DS:Chitosan	1:1	288.00	278.48	295.12	143.03
DS:LAC	1:1	191.71	179.67	196.99	47.12
DS:HPMC K15M:Chitosan	1:1:1	270.96	260.53	285.72	161.63

[a] ΔH_f corr = ΔH_f obs/% DS in sample × 100 (Vueba et al., 2005a).

both the onset and the drug melting temperatures (Table 2).

In general, the thermograms of solid state drug/excipient mixtures allow the detection of interactions between the components. However, some authors recognise that the occurrence of physical or chemical interactions does not necessarily indicate an incompatibility (Vueba at al., 2005a). Additionally, they agree that a change observed in the DSC curves is an unambiguous proof of interaction between drug and excipients (Balestrieri et al., 1996).

Raman spectroscopy

Raman spectroscopy has been widely applied to evaluate drug-excipient compatibility in pre-formulation studies (Marques et al., 2002; Vueba et al., 2006, 2008; Santos et al., 2012), and was presently used to detect solid-state interactions among DS and the excipients considered in the tested formulations. A 1:1 *(w:w)* drug:excipient ratio was chosen, as this is known to take advantage of the probability of occurrence intermolecular interactions.

The detailed assignment of the DS Raman bands has been previously proposed by Iliescu et al. (2004). The spectrum presents intense and well-defined features, most of them directly correlated to specific groups within the molecule, thus, allowing an objective identification of those involved in intermolecular interactions.

Figures 2a and 3a comprise the Raman spectra of the DS:HPMC K15M and DS:LAC physical mixtures, respectively, after one week of mixture preparation. The mixtures containing chitosan exhibited a very strong fluorescence background (Figure not shown) making it impossible to obtain its Raman spectrum with the available Ar^{+} laser.

In order to detect changes in the DS molecule upon mixing with HPMC K15M or LAC, the spectra of the excipients (Figures 2b and 3b) were subtracted from those of the physical mixtures, yielding the "DS changed" spectra (Figures 2c and 3c). The similarity between the latter and the spectrum of pure DS (Figures 2d and 3d) is quite remarkable. However, careful inspection allows the detection of some subtle differences, highlighted in

Figure 4 obtained through subtraction of the DS spectrum (Figures 2d or 3d) from the "DS changed" spectra (Figures 2c and 3c). The new signals at 267 and 360 cm^{-1} (a shoulder of the 367 cm^{-1} band), could be assigned to O-C-O[-] deformation modes of the DS interacting with the excipients, either HPMC K15M (Figure 4a) or LAC (Figure 4b). The difference signals at 1049 cm^{-1} (Figures 4c and 4d) and around 1600 cm^{-1} (Figures 4e and f), mainly assigned to changes in the O-C-O[-] stretching modes intensities, corroborate this interpretation. No other spectral changes were observed. In particular, no evidences of either DS conformational equilibrium modification or polymorphism were detected.

The observed changes in the DS Raman spectra were thus related with the interaction between the diclofenac COO[-] functional group and the excipients. However, these spectroscopic results support the absence of significant intermolecular close contacts that could eventually lead to an incompatibility between the drug and the different formulation components.

Assay of DS in matrix tablets

As summarised in Table 3, evaluation of the hydrophilic matrix tablets containing DS showed that the drug content of all formulations ranged from 95.0 to 97.1% of the defined, which evidences a content uniformity. The differences in the mean values among the treatment groups are not large enough to exclude the possibility of arising from random sampling variability and the observed difference were not statistically significant (F = 1.45; P = 0.27). These values conform to USP 34 (2011) norms concerning DS delayed release tablets, which require an even amount of drug in all formulations (from 90.0 to 110.0% of the labelled amount).

Characterisation of the tablets

Table 4 shows several parameters, namely, mean values, standard deviation (SD), relative standard deviation (RSD), median, minimum and maximum values of the formulations of DS matrix tablets concerning to
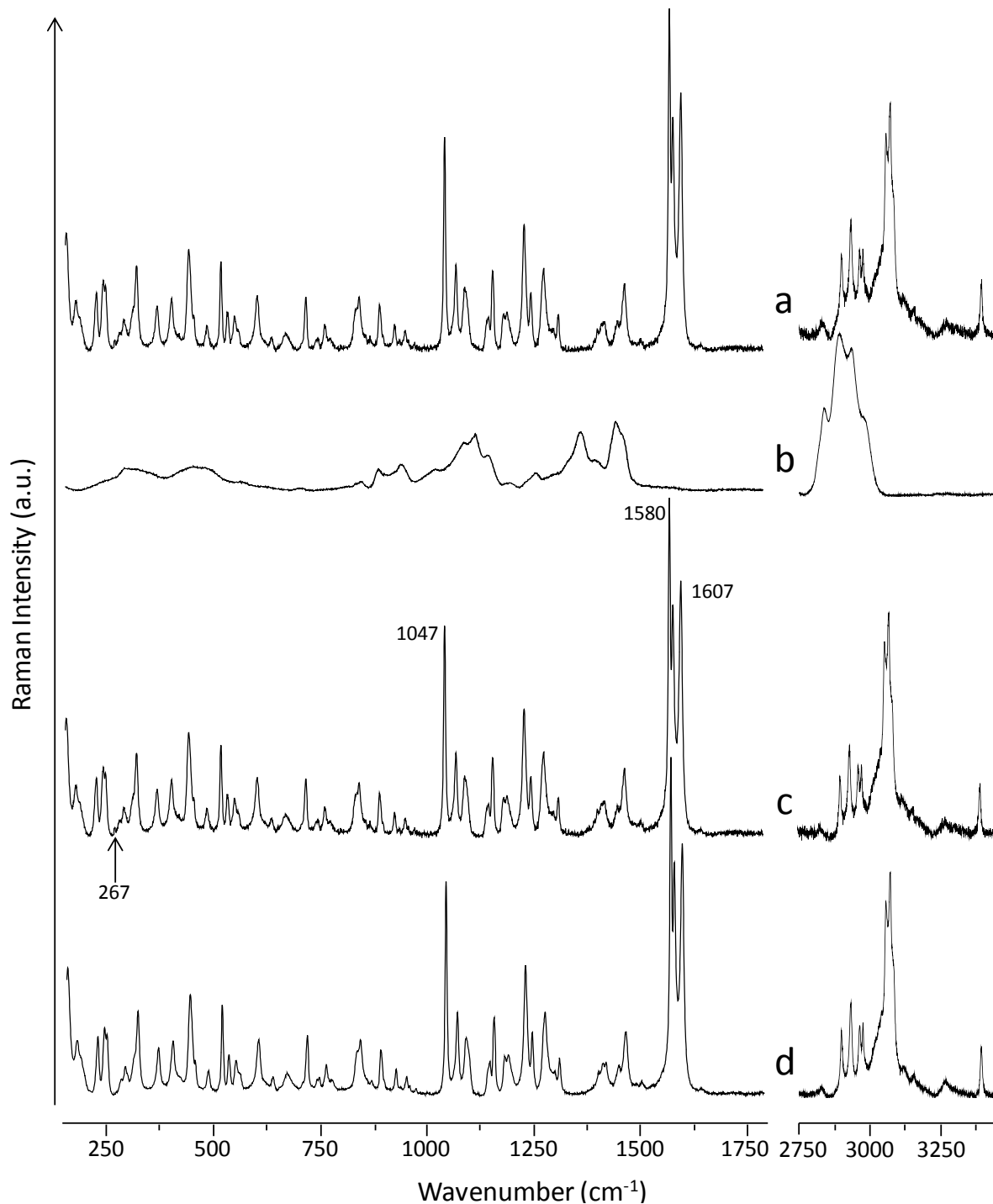
Figure 2. Raman spectra, in the 150 to 1800 and 2750 to 3450 cm^{-1} regions, of DS:HPMC K15M physical mixture (a), pure HPMC K15M (b), the result of subtraction (c), and pure DS (d).

uniformity of mass (e.g. homogeneity). Comparing formulations F1 and F4, it was possible to verify a uniformity of tablets since SD was less than 1.0; whereas, RSD was found to be lesser to 0.4%. The variance (data not shown) of all formulations was lower than 0.1 indicating a homogenous distribution of the drug

in the matrix tablets. The maximum value for formulations F1 to F4 was 277.70 mg and the minimum value was 273.80 mg. USP 34 (2011) norms require an average weight of 250 mg or more, and therefore, not more than two tablets are permitted to deviate from the mean weight by more than 5% and none more than 10%. These four
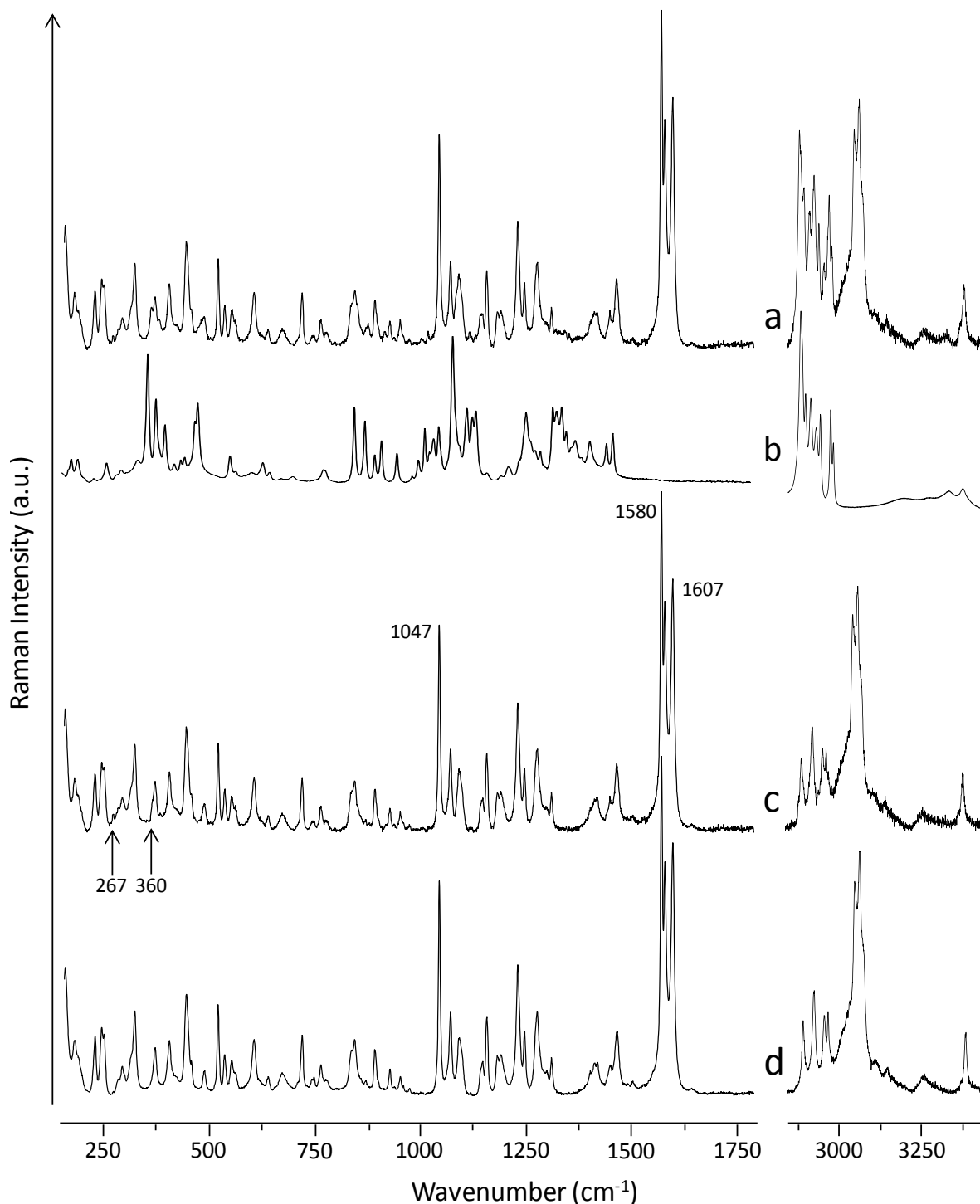
Figure 3. Raman spectra, in the 150 to 1800 and 2750 to 3450 cm^{-1} regions, of DS:LAC physical mixture (a), pure LAC (b), the result of subtraction (c), and pure DS (d).

formulations respected these criteria.

The hardness of the different formulations studied was within the range of 187.7 to 215.4 N (F = 76.920; P < 0.001), corresponding to obvious variations in the tablet tensile strength from 4.70 to 5.18 MPa (F = 14.187; P <

0.001), demonstrating a good solidity. The thickness of the tablets (Table 3) was found within the range of 2.55 to 2.65 mm (F = 46.889; P < 0.001), statistically different which may be influenced by the properties of each polymer. A lower percentage of friability is synonymous of
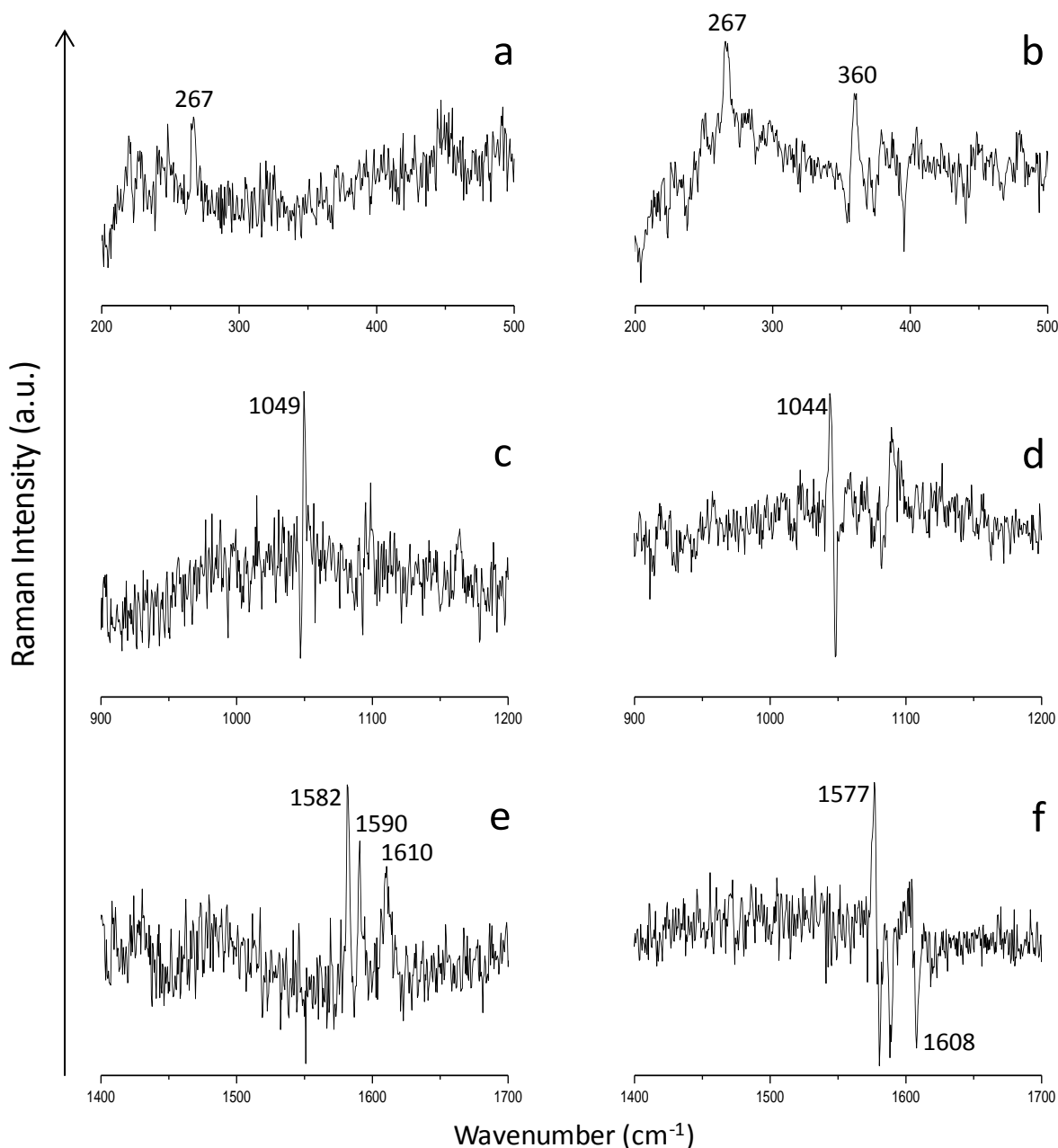
Figure 4. Different regions of the difference Raman spectra between the "DS changed" spectra and DS spectrum (see text) for: the HPMC K15M system (a), (c), (e); and LAC system (b), (d), and (f).

tablets compactness. Hence, these four formulations presented very similar compactibility since the results showed friability values in the interval of 0.10 to 0.23%, indicating that all formulations lie within the USP 34 (2011) limits.

Swelling studies

Hydrophilic polymer substances are well known to play a significant role in the swelling process of matrix tablets

(Nerurkar et al., 2005). The swelling rate, and thus the formation of a continuous gel layer, was found to be strongly dependent on both polymer hydration speed and viscosity grade (Roy and Rohera, 2002). Moreover, the polymer hydration must be fast enough to allow the formation of the gel layer before the contents of the matrix tablet; in particular, the carried drug can dissolve. In this work, swelling studies were performed in order to assess the effect of the distinct formulations on the swelling process. When a matrix is immersed in a dissolution medium, wetting occurs, first at the surface

Table 3. Physical characterisation of DS matrix tablets.

Formulation	Drug content (mg)	Hardness (N)	Tensile strength (MPa)	Thickness (mm)	Friability (%)
	n=5	n=10	n=10	n=10	n=20
F1	95.04±0.44	187.70±4.59	4.694±0.223	2.55±0.01	0.21
F2	96.08±0.62	215.40±4.42	5.182±0.020	2.65±0.01	0.10
F3	97.11±1.31	202.40±3.80	4.946±0.183	2.61±0.02	0.15
F4	96.31±2.78	208.60±4.06	5.100±0.215	2.62±0.03	0.23

[a]n is the number of measurements.

Table 4. Descriptive statistics parameters of the formulations of DS tablets relating to the uniformity of mass (n = 30).

| Formulation	Average (mg)	SD (mg)	RSD (%)	Median (mg)	Minimum (mg)	Maximum (mg)
F1	275.11	0.85	0.31	274.80	273.80	277.20
F2	275.69	0.93	0.34	275.50	274.20	277.60
F3	274.85	0.50	0.18	274.80	274.10	276.50
F4	275.92	0.66	0.24	275.90	274.70	277.70
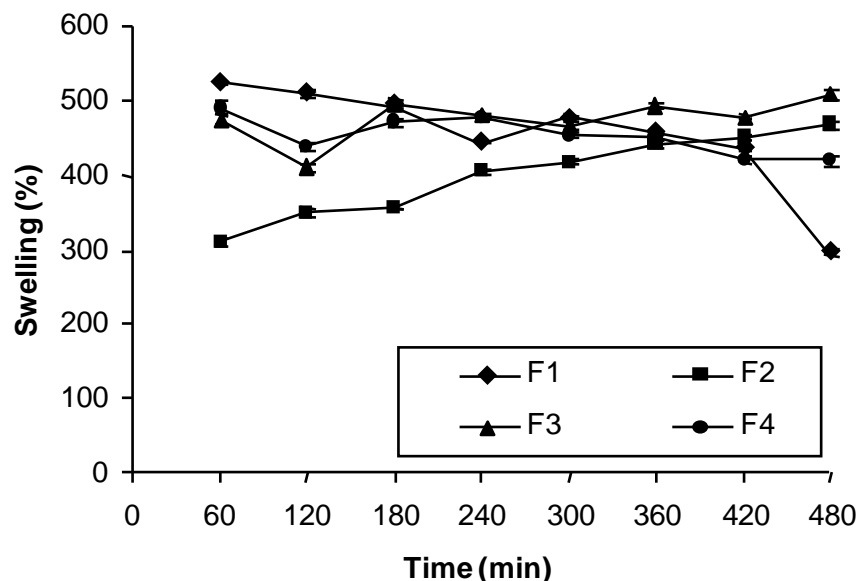
Figure 5. Graphical representation of the water uptake versus time of DS matrix tablets formulations containing different concentrations of HPMC K15M and binary mixtures of HPMC K15M with chitosan (Table 1).

and then progressing into the matrix (Sriamornsak et al., 2007). The results of the swelling studies are gathered in Figure 5. Formulations F1, F3, and F4 attained their maximum hydration degree (between 400 and 500%) during the first hour. However, while F3 and F4 roughly retain this hydration level; for F1, a progressive decrease was observed over the next 7 h. This may be explained by the low amount of the polymer and the higher quantity of LAC on the F1 formulation (Table 1). These results are in accordance with previous reported studies on the

HPMC swelling behaviour (Borguist et al., 2006), explained by the presence of the substituent groups which, through interaction with water molecules, lead to an increased swelling. Formulation F2, in turn, due to the higher quantity of HPMC K15M and low amount of LAC, displayed a slower water uptake, but it attains the same level hydration after at 6 h. The polymer surface swells to form a continuous gel layer and the matrix size progressively increases (Gao et al., 1996).

The similarity observed for the swelling behaviour of F3
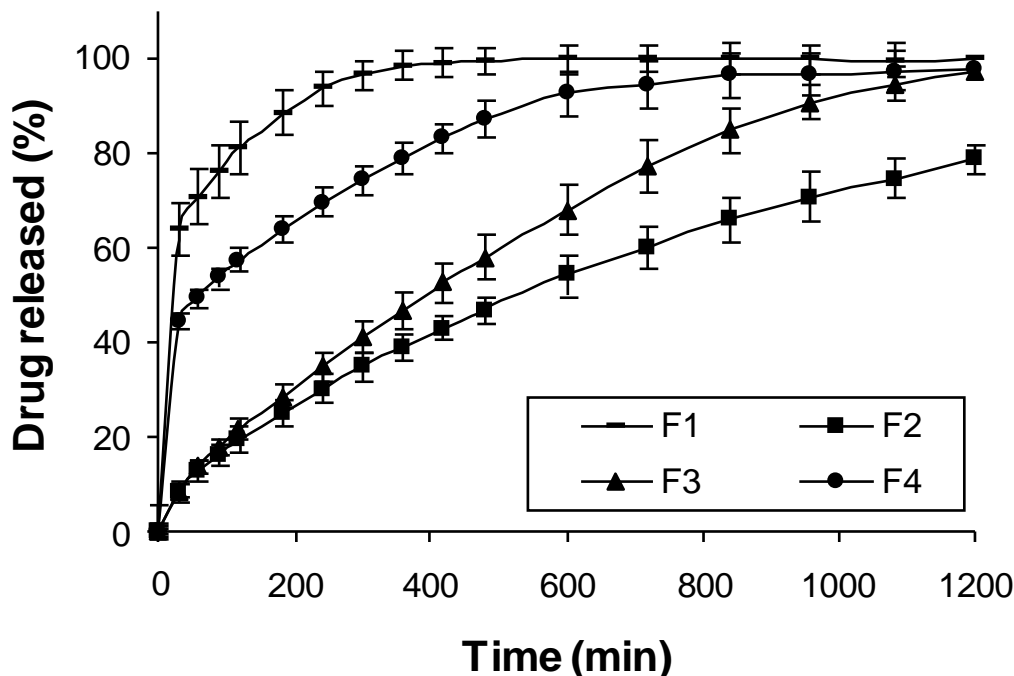
Figure 6. Dissolution profiles of DS matrix tablets formulations containing different concentrations of HPMC K15M and binary mixtures of HPMC K15M with chitosan (Table 1).

Table 5. Experimental results according to the dissolution parameters of DS matrix tablets.

Formulation	$t_{50\%}$ (h)	DE (%)	MDT (h)[a]	AUC	[b]P (%) 20 h
F2	8.86	50.27 ± 1.67	6.26 ± 0.13	1005.48	78.54 ± 0.02
F3	6.55	62.77 ± 1.48	4.53 ± 0.07	1255.38	97.06 ± 3.65
F4	1.07	83.92 ± 1.62	1.05 ± 0.05	1678.36	97.96 ± 5.03

[a]Mean ± SD (6 measurements). [b]P = percentage of DS dissolved at 20 h.

and F4 is quite interesting. In fact, the two formulations differ in the partial substitution of HPMC K15M (present in F3) by chitosan, keeping the LAC amount constant. The results now obtained may be an indication that the swelling characteristics of HPMC K15M and chitosan are comparable.

Drug release analysis

The DS release profiles from matrix tablets are as shown in Figure 6. In the case of the F1 formulation, more than 80% of the drug was released in about 3 h, due to the lower amount of polymer which leads to tablet disintegration (Abdelbary and Tadros, 2008). The slowest drug release, in turn, is observed for F2 (Figure 5), due to its highest polymer content. The gel layer viscosity increased, which results in a higher resistance to both dissolution and erosion (Borguist et al., 2006).

Concerning formulations F3 and F4, the presence of chitosan was found to have a marked effect on the drug release profile (Figure 6). In particular, the release of the drug from chitosan containing formulation (F4) was quite

rapid in the first hour, but became slow after that, which may be attributed to the lower percentage of chitosan in the formulation (Akbuga, 1993). The MDT calculated values (Table 5) corroborated these observations. In effect, this parameter may be used to characterize both the drug release process and the retarding efficacy of a polymer; a higher value of MDT indicates a higher drug retarding ability of the polymer and *vice-versa*. On the other hand, DE is a dissolution parameter widely used as a significant index of drug dissolution performance. Actually, differences were detected between the calculated dissolution parameters (Table 5).

Previous work reported that Fickian diffusion through a hydrated chitosan-containing matrix is not the only mechanism that accounting for the release, as tablets with a low concentration of chitosan showed significant disintegration characteristics (Savaser et al., 2005). In fact, this quick drug release was attributed to the rapid chitosan dissolution, even in the presence of an interaction with a negatively charged (acidic) drug, such as DS (Puttipipatkhachorn et al., 2001). Burst release is

Table 6. Results of fitting the DS release data for F2 and F3 formulations to different kinetic equations*.

Formulation	Zero order		First order		Higuchi		Korsmeyer–Peppas			Peppas–Sahlin		
	k_0 (% min^{-1})	R^2	k_1 (min^{-1})	R^2	k_H (% min$^{-1/2}$)	R^2	k_{KP} (min^{-n})	n	R^2	k_F (min$^{-0.447}$)	k_R (min$^{-0.894}$)	R^2
F2	0.09501 (0.00115)	0.8285	0.00135 (0.00001)	0.9667	2.11984 (0.01033)	0.9717	0.00981 (0.00004)	0.6259 (0.0006)	0.9999	0.01647 (0.00012)	0.00082 (0.00001)	0.9993
F3	0.13036 (0.00143)	0.9123	0.00181 (0.00001)	0.9895	2.41834 (0.02222)	0.9385	0.00735 (0.00004)	0.7062 (0.0010)	0.9999	0.01262 (0.00007)	0.00152 (0.00001)	0.9999

*Values in parenthesis mean standard deviation; R^2 is the coefficient of determination.

often observed prior to or during development of a diffusion barrier capable of controlling the penetration of the dissolution medium and the drug diffusion process (Huang and Brazel, 2001).

This behaviour of low chitosan content matrices, combined with the HPMC retarding release ability, may be used to design formulations with a more rapid initial release, so that the drug reaches a suitable plasma level, followed by a slower release to maintain the desired (therapeutic, non-toxic) level.

Kinetic mechanism

DS (pK_a = 4.0) has a very poor solubility in water and aqueous acidic conditions, which gradually increases when the pH is raised above 6, and becomes freely soluble at pH = 7 (Liu et al., 1995). During this study, the experimental conditions were established for a pH = 6.8 phosphate buffer solution, used as the dissolution medium. Thus, both diffusion and erosion could contribute to the drug release process from the matrix tablets. In fact, in polymeric swellable hydrophilic matrices similar to the ones considered, water-soluble drugs are released mainly by diffusion across the gel layer, whilst

barely water-soluble drugs are predominantly released by attrition mechanisms (Vazquez et al., 1992). Even if some processes could be characterised as either purely diffusional or purely erosion-controlled, several others could only be rationalised as being due to a coupling of both (Katzhendler et al., 2000). The use of Korsmeyer-Peppas (Equation 7), and particularly the interpretation of the release exponent values (n), allows the getting of an insight into the balance between these mechanisms (Costa and Sousa Lobo, 2001).

However, both F1 and F4 formulations must be discarded from this kind of analysis due to their very fast initial drug release, *ca.* 50 and 40% of the drug was released during the first 10 min, respectively (Figure 6).

For the F2 and F3 formulations, n was found to be equal to 0.626 and 0.706, respectively (Table 6), indicating an anomalous (non-Fickian) mechanism transport, best described by a coupling of diffusion and macromolecular relaxation processes. The difference between these two values confirms the previously mentioned dependence of the DS release mechanism on the HPMC K15M concentration.

To assess the relative contribution of Fickian diffusion and polymer relaxation (erosion)

mechanisms, over the first 60% of the drug release, for formulations F2 and F3, the values of the Fickian constant, k_F, and the relaxational constant, k_R, were calculated according Equation 8 (Table 6). The estimated contributions of the two mechanisms are presented graphically in Figure 7.

The release rate constant values (Table 6) permits the conclusion that for both the F2 and F3 formulations, diffusion was the primary release mechanism and polymer relaxation was the secondary one.

In the case of the F2 formulation, the Fickian mechanism predominates during the first 14 h of the release process (Figure 7a). Such behaviour is very well explained, since the HPMC polymer concentration in the formulation is around 36%. Consequently, an increase of the viscous gel layer around the matrix creates a longer path length for diffusion (Sujja-areevath et al., 1998).

For the F3 formulation, in turn, the decrease of hydrophilic polymer amount to *ca.* 31%, coupled to an equalizer increase of LAC, causes a drastic change in the diffusion/erosion mechanisms balance (Figure 7b). In fact, for this formulation, the diffusion dominates only during the first 2 h of the dissolution time, while polymer relaxation rapidly increases and hence becomes
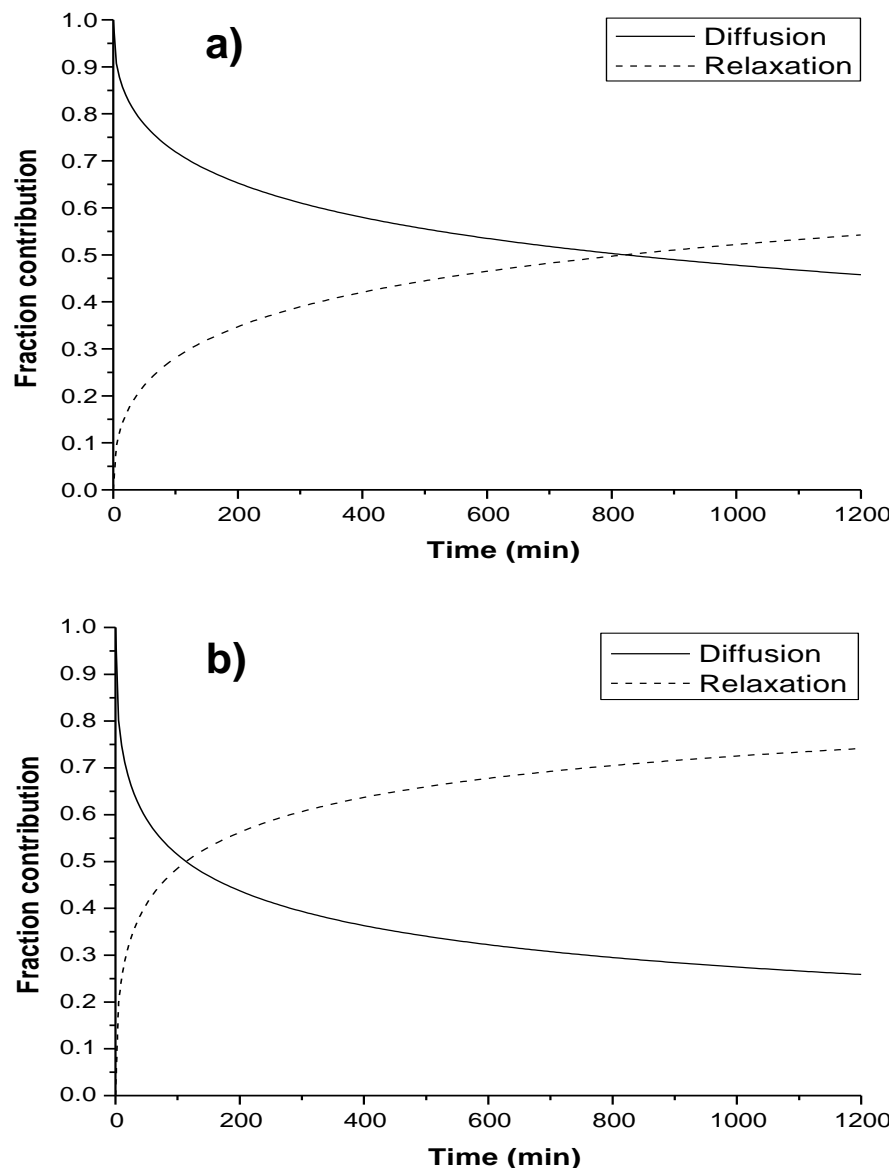
Figure 7. Fraction contribution of the Fickian diffusion and the erosion mechanisms (a) for formulation F2 (k_F = 0.01647 min$^{-0.447}$ and k_R = 0.00082 min$^{-0.894}$) and (b) for formulation F3 (k_F = 0.01262 min$^{-0.447}$ and k_R = 0.00152 min$^{-0.894}$).

predominant for the rest of the process. The contribution of polymer relaxation to the drug release mechanism was found to increase as the HPMC K15M concentration decreased as evidenced by the magnitude of the corresponding k_R values (Table 6). This behaviour confirms the previously mentioned dependence of the DS release mechanism on the polymer concentration.

Furthermore, these results are consistent with the Korsmeyer-Peppas exponent calculated values indicating an anomalous transport, but in which the Fickian contribution is greater for F2 formulation, corresponding to a smaller value of n (0.626) when compared with that obtained for F3 (n = 0.706).

Conclusions

Matrix formulations containing DS and different concentrations of HPMC K15M or HPMC K15M/chitosan were assessed for their drug content, weight uniformity, hardness, thickness, tensile strength, friability, porosity, swelling, and drug release performance. From the DSC thermograms of the mixtures tested, it was possible to detect some drug:excipient interactions. Raman spectroscopy data allowed the conclusion that these interactions occur mainly between the diclofenac COO$^-$ functional group and the polymers. However, no intermolecular close contacts, which could eventually

lead to an incompatibility between the drug and the different formulation components, were detected. Hence, the selected excipients are suitable for the preparation of tablet formulations.

Regarding the DS release from matrix formulations, the results presently obtained indicate that low concentrations of HPMC K15M do not control the release of the drug. The release mechanisms of DS from formulations F2, F3, and F4 were evaluated using, among others, the Korsmeyer-Peppas kinetic model. Only for F2 and F3, could a clear fitting be obtained, reflecting an anomalous transport mechanism. A decrease in polymer concentration was found to lead to a marked change in the drug release characteristics, that is, in the diffusion/erosion balance, assessed by the Peppas-Sahlin model.

For the chitosan containing formulation (F4), a burst release is detected prior to the drug diffusion through the matrix. This behaviour of low chitosan content matrices, combined with the HPMC K15M retarding release ability, may be used to design formulations with a more rapid initial release, so that the drug reaches a suitable plasma level, followed by a slower release to maintain the desired therapeutic level.

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