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Fully automated analytical method for codeine quantification in human plasma using on-line solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple, sensitive and fully automated analytical method for the analysis of codeine in human plasma is presented. Samples are added with oxycodone, used as internal standard (I.S.), and directly loaded in the autosampler tray. An on-line sample clean-up system based on solid-phase extraction (SPE) cartridges (Bond-Elut C_2, 20 mg) and valve switching (Prospekt) is used. Isocratic elution improved reproducibility and allowed the recirculation of the mobile phase. A Hypersil BDS C_{18}, 3 μm, 10×0.46 cm column was used and detection was done by UV monitoring at 212 nm. Retention times of norcodeine (codeine metabolite), codeine and oxycodone (I.S.) were 5.5, 6.4 and 9.1 min, respectively. Morphine was left to elute in the chromatographic front. Detection limit for codeine was 0.5 μg l^{-1} and inter-assay precision (expressed as relative standard deviation) and accuracy (expressed as relative error) measured at 2 μg l^{-1} were 5.03% and 1.82%. Calibration range was 2–140 μg l^{-1}.

Keywords: Codeine; Norcodeine

1. Introduction

Codeine is widely used as a good analgesic and antitussive agent [1–5]. New pharmaceutical preparations appearing require fast, sensitive and simple (cheap) bioanalytical methods for the quantitative determination of codeine in human plasma in bioavailability or bioequivalence studies.

Different chromatographic methods have been previously reported for the analysis of codeine and/or its metabolites in biological fluids. High-performance liquid chromatography (HPLC) has been the preferred technique in most of the applications using a variety of detection methods such as ultraviolet [6,7], fluorescence [7–9] and electrochemical [10–12] or a combination of them in series [7]. Others used gas chromatography (GC) with nitrogenous specific detection [13–15] or even GC–mass spectrometry (MS) after a derivatisation step [15–19]. Sample preparation previous to instrumental analysis was based on liquid–liquid extraction procedures which are time consuming and solvent-usage intensive [7,8,10,13,14,18]. More recently, solid-phase extraction (SPE) with different stationary phases (hydrophobic, cationic exchange or mixed) has become the method of choice [6,9,11,12,16,17]. Dialysis has also been used for sample clean-up [15].

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Nevertheless, all of them are off-line methods and require a set of manual operations before the sample can be instrumentally analysed. Despite the variety of techniques and combinations used, limits of quantification have almost always been above 10 μg l^{-1}. Other factors like interferences appearing near the peaks of interest or baseline instability, dramatically limited their use when low therapeutical doses of codeine (e.g., 30 mg of codeine phosphate) are orally administered. After such a dose, peak plasma concentrations (C_{max}) of codeine can be as low as 40 μg l^{-1}. Codeine has a short half life (~2 h) so that monitoring plasmatic concentrations up to at least 12 h after dosing requires lowering the currently achieved quantification limits and the use of rugged and reproducible methods allowing the analysis of many samples per day without loosing chromatographic behaviour or sensitivity. New possibilities of fully automating SPE methods through valve-switching devices and automatic cartridge exchange together with the use of fast chromatographic analysis will allow both lowering the detection limits and a considerable increase in reproducibility through a nearly null human intervention.

Herein we present the development, validation and retrospective evaluation of a fully automated analytical method for the determination of codeine in human plasma based on on-line valve-switching SPE via a Prospekt system followed by fast HPLC separation and UV detection at 212 nm, the maximum of the UV spectrum of codeine under the conditions of analysis.

2. Experimental

2.1. Chemicals and reagents

Codeine (7,8-dehydro-4,5-epoxy-6-hydroxy-3-methoxy-*N*-methylmorphinan) phosphate hemihydrate was kindly supplied by Laboratorios GELOS (Esplugues de Llobregat, Spain). Oxycodone (4,5-epoxy-14-hydroxy-3-methoxy-*N*-methyl-6-oxomorphinan) hydrochloride and norcodeine (7,8-dehydro-4,5-epoxy-6-hydroxy-3-methoxymorphinan) were purchased from Sigma (St. Louis, MO, USA). Structures of both compounds and their UV spectra are shown in Fig. 1.

Ultra-pure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). Methanol (Scharlau, Barcelona, Spain) and acetonitrile (Merck, Darmstadt, Germany) were HPLC grade. Other reagents were analytical reagent grade. A 10 m*M* KH_2PO_4 (pH 3.0) solution was prepared by dissolving 1.36 g of KH_2PO_4 in water and making up the volume to 1 l. The solution was adjusted to pH 3.0 by dropwise addition of 0.85% H_3PO_4. A 1 m*M* KH_2PO_4 (pH 3.0) solution was prepared by 1/10 aqueous dilution of the former.

SPE cartridges (10×2 mm I.D.), Bond Elute C_2 (40 μm, 20 mg) were purchased from Spark Holland (Emmen, The Netherlands).

2.2. Stock and working solutions

Stock solutions (1 g l^{-1}) of codeine and oxycodone (I.S.) were prepared in methanol. Dilute stock solutions at 100, 10, 1 and 0.1 mg l^{-1} of codeine were prepared by appropriate dilutions with methanol. All solutions were stored at $T<-20^{o}C$ until used. Under this conditions, solutions are stable for more than six months. A working solution of oxycodone (0.1 mg l^{-1}) was prepared by dilution of the stock solution with water. This solution was prepared weekly and stored at 4°C.

2.3. Preparation of calibration and quality control samples

Calibration samples containing 2, 10, 40, 80 and 140 μg l^{-1} of codeine (as free base) were prepared daily for each analytical batch in checked drug-free (blank) human plasma. Suitable amounts of dilute stock solutions of codeine (1 and 0.1 mg l^{-1}) were placed in 15-ml screw-capped disposable glass tubes and evaporated to dryness under a stream of nitrogen before adding 700 μl of blank plasma.

Pooled quality control (QC) samples containing codeine were prepared at two different concentrations, 5 and 100 μg l^{-1} by adding suitable amounts of stock solutions of codeine (10 and 100 mg l^{-1}) to 25-ml volumetric flasks and taking to the volume with checked blank plasma. Once prepared, samples were divided in 700-μl aliquots in screw-capped disposable glass tubes and kept at $T<-20°C$ until analysis. Aliquots of both control samples were

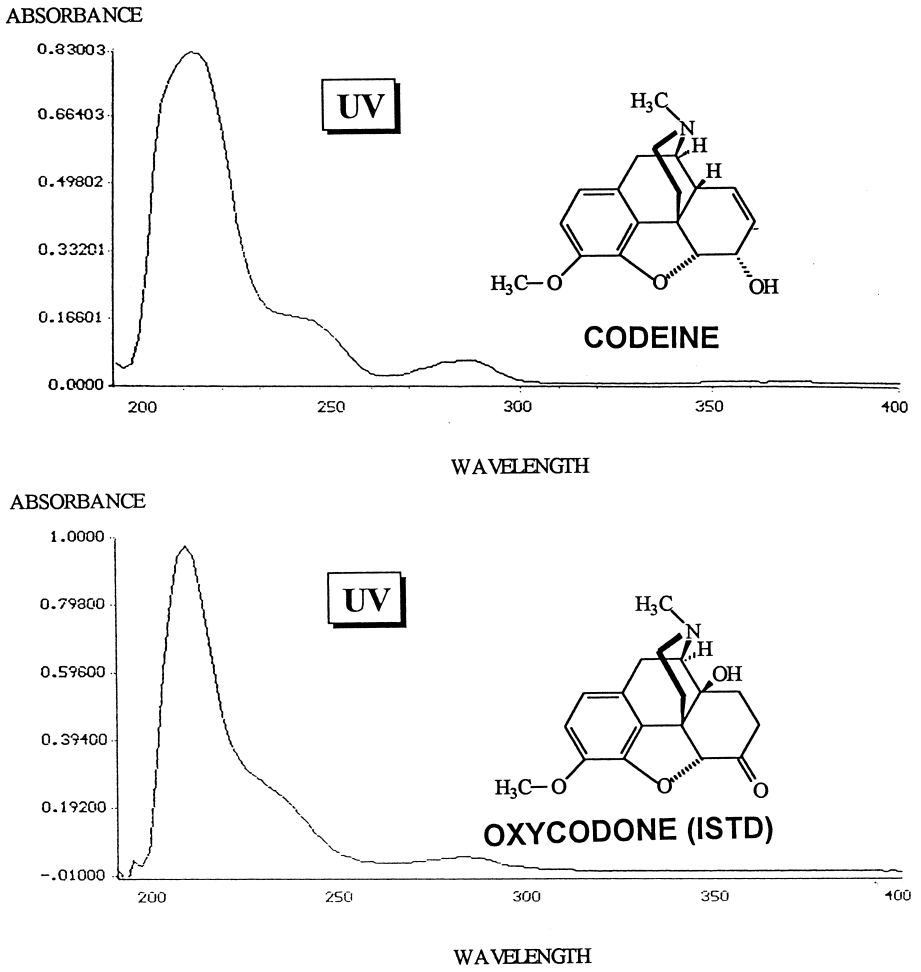

Fig. 1. Molecular structures and UV spectra of codeine and oxycodone (used as internal standard).

included in each analytical batch to check for accuracy as well as stability under storage conditions.

2.4. Instrumentation

The whole instrumental set-up is schematically shown in Fig. 2. The system consisted of three modules: a thermostatted autosampler equipped with a 2-ml sample loop (ISS-100, Perkin-Elmer, Norwalk, CT, USA), an on-line SPE extraction system with a six-port solvent delivery unit (SDU) and a Prospekt valve switching system (Spark Holland) and the HPLC apparatus with a quaternary pump (1050 series, Hewlett-Packard, Palo Alto, CA, USA) and a variable-wavelength detector (1050 series, Hewlett-Packard). Data processing was carried out using a Peak96 V.A. 02.01 software running on a personal computer and a hp3396 integrator (Hewlett-Packard).

2.5. Chromatographic conditions

The analytical column was a Hypersil BDS C_{18}, 3 μm particle size, 10×0.46 cm I.D. (Labsystems, Runcorn, UK). Isocratic elution was achieved using a mobile phase consisting of 10 m*M* KH_2PO_4 at pH 3.0—acetonitrile (93:7, v/v), which was filtered

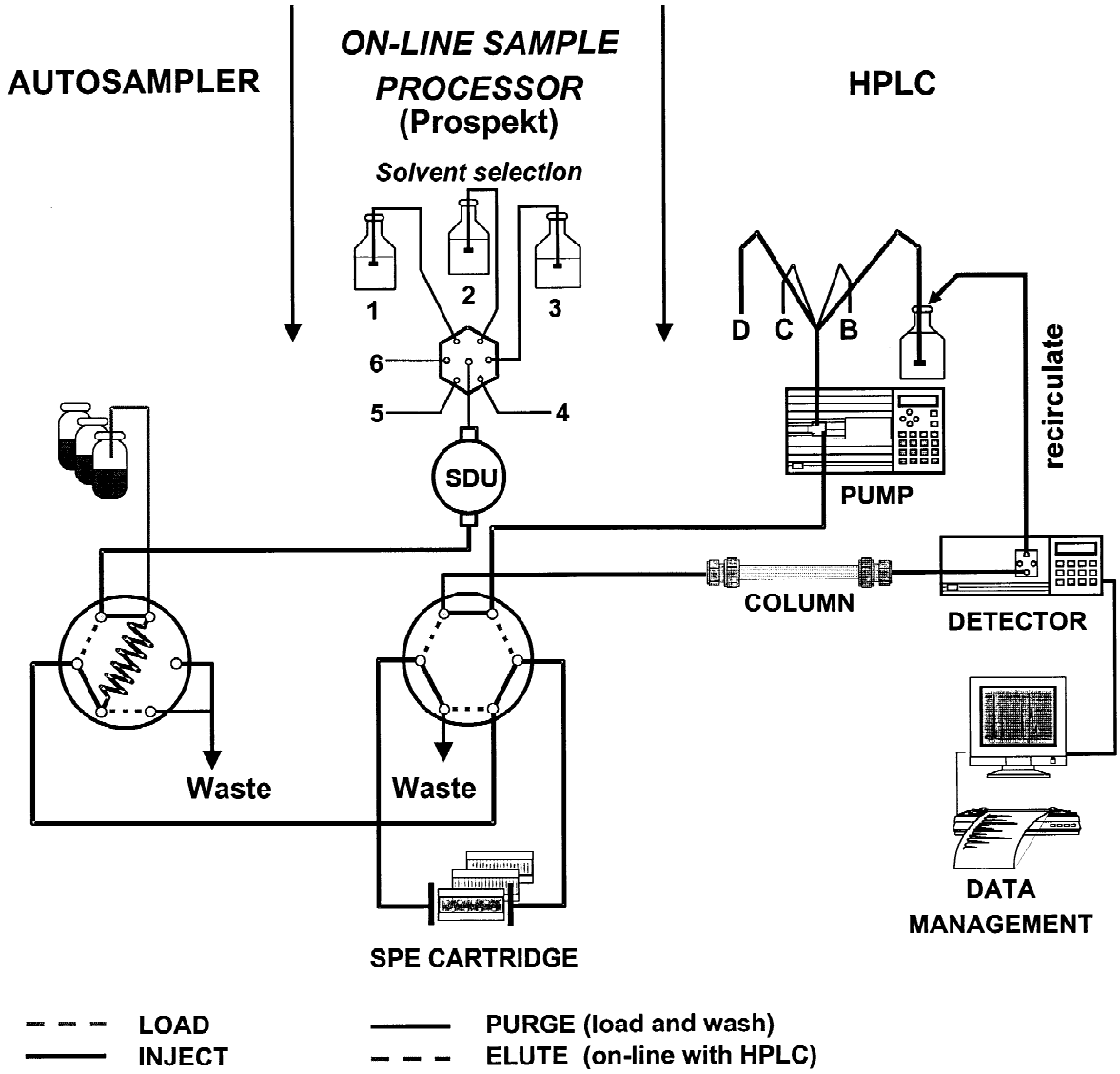

Fig. 2. Schematic representation of the instrumental system used in this work. The systems consisted of three modules: Left: a thermostatted autosampler equipped with a 2-ml sample loop. Center: an on-line SPE extraction system with a six-port solvent delivery unit (SDU) and a Prospekt valve switching system. Right: the HPLC apparatus with a quaternary pump, a variable-wavelength detector and the data processing device. Samples ''loaded'' in the autosampler loop are then ''injected'' which means transferred and retained on the SPE cartridge and then ''eluted'' to the HPLC system.

through a nylon filter membrane 47 mm O.D., 0.45 μm pore size (Hewlett-Packard) and degassed with a constant helium flow during use. Flow-rate was 1 ml min^{-1}. Detection was carried out by UV monitoring at 212 nm.

2.6. On-line sample preparation

To an aliquot of 0.7 ml of a plasma sample, 0.4 ml of the internal standard working solution was added. The mixture was vortex mixed and transferred to a

Table 1
Sequence of automatic operations of the on-line sample preparation program

Time	SDU[a] solvent	SDU flow-rate (ml/min)	Switches	Valve V1	Comments
0:00	1	2		Purge	Cartridge activation
2:00	2	2			Cartridge conditioning
4:00			Load		Beginning of injection cycle of autosampler (sample loaded into the loop)
4:01			Inject		End of injection cycle. Injection loop on-line with SPE cartridge.
4:02	3	2			Sample cleaning
4:45	2	2			Cartridge conditioning prior to elution
8:00			Start	Elute	Cartridge on-line with HPLC column Start acquisition
9:00	1	1		Purge	Cartridge regeneration

[a] SDU solvents: 1=MeOH, 2=water, 3=1 m*M* KH_2PO_4, pH 3.0—MeOH (5:1).

2-ml injection vial which was then loaded into the autosampler tray (thermostatted at 4°C). Sample was ready for instrumental analysis.

0.8 ml of the content of the injection vials were injected in the system following the sequence of automatic operations described in Table 1. Basically, the sample preparation process consisted in an activation of the cartridge stationary phase with methanol and then conditioning with water. Sample was then applied (transferred from the loop of the autosampler by means of a water flow pumped by the SDU. Under these conditions, compounds of interest are retained in the cartridge. Sample is ready for chromatographic analysis. With the start signal, a valve switch put the cartridge in contact with the chromatographic mobile phase which is able to elute the analytes to the analytical column. Although the SPE cartridge was exchanged after each injection, a last step for cleaning–regeneration of the cartridge was added to the method. Under this conditions, cartridges could be re-used, later on, at least up to three times without noticeable changes in behaviour (i.e., identical extraction recovery, peak shape, no appearance of extra-ghost peaks, etc.), hence significantly reducing the cost of analysis.

3. Results and discussion

The method was validated following a protocol consisting of three assays carried out on different days. The method was tested for selectivity, recovery, linearity, precision, accuracy and limit of detection and quantification. Retrospective data is also given after 15 sample batches analysed. Oxycodone was chosen as internal standard given its similar chemical structure and UV spectrum as well as its chromatographic behaviour, eluting well separated after codeine. Other physicochemical properties like recovery and stability were considered as well. Fig. 3 shows representative chromatograms of blank plasma, a calibration sample at 2 $\mu g\ l^{-1}$ of codeine and a sample corresponding to a real sample from a volunteer who received 30 mg of codeine phosphate. Concentrations of codeine could be monitored for at least 12 h after the administration.

3.1. Selectivity

The selectivity of the method was tested by analysing blank plasma samples from different sources and checking for the absence of any interfering peak at the retention time of the compounds of interest, codeine and oxycodone (i.e., 6.4 and 9.1 min, respectively). The method showed to be specific for those substances as well as for norcodeine (codeine metabolite) eluting right before codeine (at 5.5 min) as a peak baseline separated. Morphine, another codeine metabolite, eluted with the chromatographic front (at 1.5 min) under the chosen chromatographic conditions. Under routine operation, a total of 24 different sources of blank plasma samples were checked showing the complete absence of interferences under the conditions described.

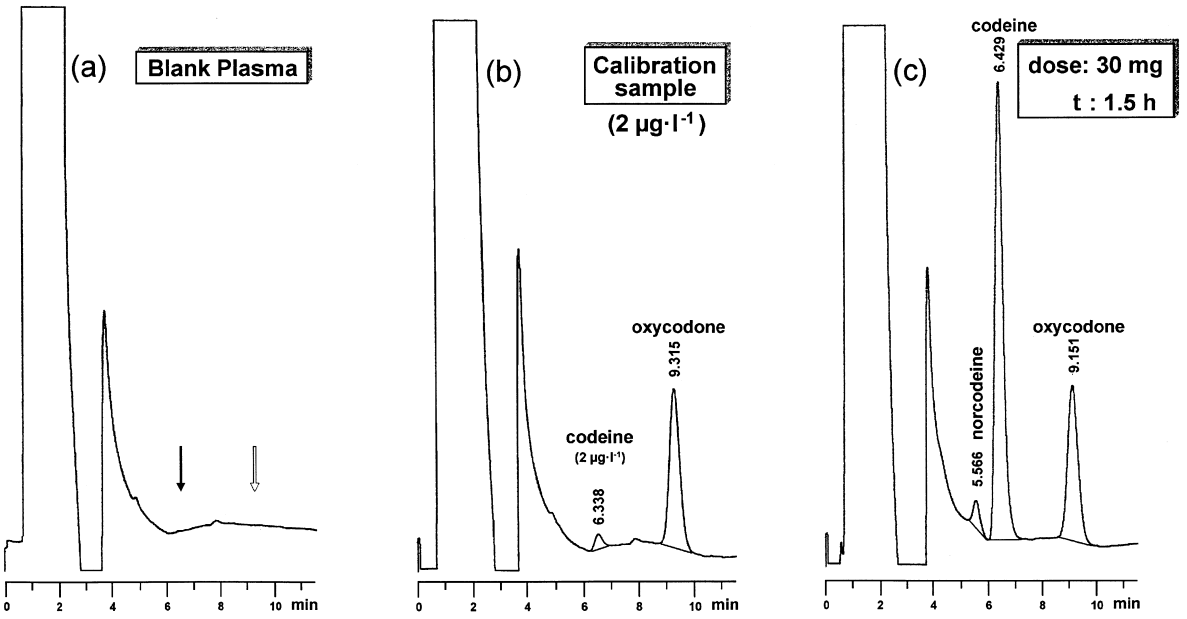

Fig. 3. Representative chromatograms obtained using the method described. (a) Blank plasma. Arrows indicate the positions where codeine and oxycodone should appear. (b) Calibration sample at the concentration of 2 μg l^{-1}. (c) Sample corresponding to a volunteer who received an oral dose of 30 mg of codeine phosphate.

3.2. Recovery

For the determination of recoveries, it was necessary to modify the connection through the Prospekt valve switching system so that the injector was connected on-line with the analytical pump and direct injections (by-passing the SPE cartridge system) were possible. Under these conditions, amounts identical to those corresponding to the calibration samples of 2, 40 and 140 μg l^{-1} were directly injected ($n=6$ each) and the areas of the peaks taken as 100%. The comparison of the areas obtained with those of the equivalent chromatograms found for the samples of the calibration curve on the same date gave mean recoveries of 91.9% for codeine and 90.6% for oxycodone without any significant influence of the concentration.

3.3. Linearity

For the study of linearity, calibration curves were prepared covering the concentration range expected in real samples (2, 10, 40, 80 and 140 μg l^{-1}). Regression analysis was performed using GraFit ver. 3.01 for windows (Erithacus Software, Staines, UK) plotting concentrations of codeine, as independent variable versus area ratios (codeine over oxycodone) as dependent variable. To correct for the heteroscedasticity of the data, the model was weighted by the inverse of the variance. A linear correlation was found, with determination coefficients better than 0.996 in all cases. After routine use of the method, the relative standard deviation (RSD) of the slope was 5.05% (after 15 calibration curves) and mean coefficient of determination (r^2) was 0.9974.

3.4. Precision and accuracy

The instrumental precision is expressed as the RSD (%) of the peak area ratios obtained after repeated direct injections of aqueous solutions at different concentrations. Results obtained were 4.6%, at 2 μg l^{-1}, 0.93% at 40 μg l^{-1} and 1.27% at 140 μg l^{-1} ($n=6$ in all cases).

Table 2
Intra-assay and retrospective evaluation (inter-assay) precision and accuracy found in the determination of codeine using the described method

Concentration ($\mu g\ l^{-1}$)	Intra-assay			Inter-assay (retrospective evaluation)		
	N	Precision (RSD, %)	Accuracy (RE, %)	*N*	Precision (RSD, %)	Accuracy (RE, %)
2	6	6.96	0.60	33	5.03	1.82
10				30	3.85	−2.10
40	6	0.75	0.31	34	2.34	0.54
80				30	4.01	1.68
140	6	3.61	−0.40	34	3.51	0.69

Intra-assay precision of the method is expressed as the RSD (%) of the estimated concentration values obtained for samples along the calibration range analysed in one assay ($n=6$). Inter-assay precision is given for the retrospective value obtained considering all the assays carried out during more than one month of routine use of the method ($n>30$). Precision values in the retrospective inter-assay evaluation were around 5% or lower showing the reproducibility and robustness of the method along the period of time investigated.

Accuracy is expressed as the relative error (RE) in the estimation of each concentration. As before, inter-assay accuracy is given for the retrospective evaluation of all values obtained. Using the regression model described, relative errors found in the estimated concentrations were always near or below 2%. Results for precision and accuracy are shown in Table 2.

The absence of human operations and the stability of the UV detector are key factors in achieving the reported reproducibility.

Quality control samples were prepared in pooled blank human plasma at the concentrations of 5 $\mu g\ l^{-1}$ (control ''low'') and 100 $\mu g\ l^{-1}$ (control ''high'') and analysed in parallel with each analytical batch. Results obtained (Table 3) show also the robustness of the method along the time of use.

3.5. Limit of detection and quantification

The limits of detection and quantification are defined, for the purpose of this method, as the concentrations for which a signal-to-noise ratio of 3 or 10 (respectively) are obtained. The noise is estimated by the standard deviation of the concentration values obtained for the calibration samples at the lowest concentration of the curve (2 $\mu g\ l^{-1}$). This definition tends to over-estimate the magnitude of the noise ensuring a conservative estimation of the limit of quantification. Under this conditions the limit of detection was estimated as 0.47 $\mu g\ l^{-1}$ and the limit of quantification as 1.56 $\mu g\ l^{-1}$ showing the suitability of the calibration range established from 2 $\mu g\ l^{-1}$.

Fig. 4 shows the pharmacokinetic profile of codeine obtained after oral administration of 30 mg of codeine phosphate to a healthy volunteer. As can

Table 3
Results obtained for the quality control samples analysed in parallel with all the analytical batches

Quality control	Concentration ($\mu g\ l^{-1}$)	*N*	Estimated concentration ($\mu g\ l^{-1}$)	Precision (RSD, %)	Accuracy (RE, %)
Low	5	18	5.1	7.16	2.78
High	100	18	100.1	5.06	0.07

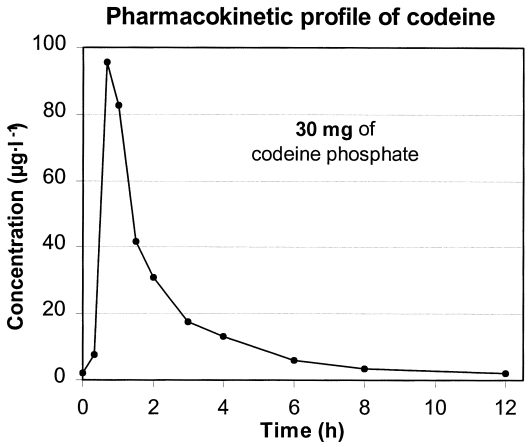

Fig. 4. Pharmacokinetic profile of codeine obtained after administration of an oral dose of 30 mg of codeine phosphate to a healthy volunteer. The concentration of the lower level of the calibration range (i.e., 2 μg l^{-1}) has been assigned to samples in which no codeine was detected (i.e., sample corresponding to 0 h).

be seen, plasma concentrations could be monitored for up to 12 h after administration.

4. Conclusions

Automation of sample preparation through the use of on-line SPE with automatic valve switching and cartridge exchange has proved to be an excellent approach for the unattended analysis of human plasma samples. Minimising human intervention makes the method easy to apply and improves reproducibility and accuracy.

A method was designed to get a fast chromatographic separation of codeine from the possible interfering substances (e.g., norcodeine or morphine) appearing in plasma samples. Furthermore, samples could be automatically prepared while running the chromatographic analysis of the previous one, so that continuous analysis could be done. Using isocratic elution, mobile phase could be recirculated, saving solvents. On the other hand, re-use of SPE cartridges after automatic cleaning as part of the method substantially reduced the cost of the analysis.

For the purpose of the method, a low limit of detection was needed forcing the selection of the wavelength to maximise the response. 212 nm was chosen for detection even though it is very unspecific and interferences could easily appear. Using the described automatic on-line sample clean-up, no interferences or late eluting peaks appeared after hundreds of samples analysed showing the quality and validity of this approach.

Other opiates like dihydrocodeine, hydromorphone, hydrocodone, etc., could be easily analysed by this technique by just applying simple modifications.

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