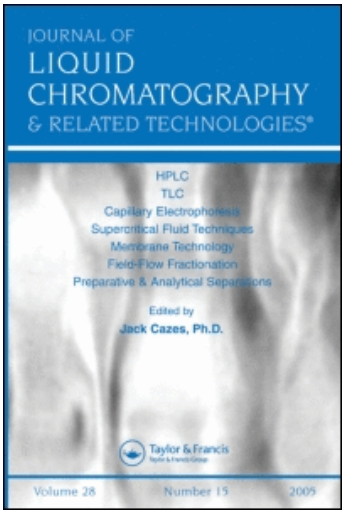

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I. Carretero[a]; M. Morell[b]; J. J. Laserna[a]
[a] Department of Analytical Chemistry Faculty of Sciences, University of Málaga, Málaga, Spain [b] Department of Biochemistry Faculty of Medicine, University of Málaga, Málaga, Spain

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DETERMINATION OF DIPYRONE METABOLITES IN HUMAN PLASMA BY MICELLAR LIQUID CHROMATOGRAPHY

I. CARRETERO[1], M. MORELL[2], AND J. J. LASERNA[1]
[1]*Department of Analytical Chemistry*
Faculty of Sciences
University of Málaga
29071 Málaga, Spain
[2]*Department of Biochemistry*
Faculty of Medicine
University of Málaga
29071 Málaga, Spain

ABSTRACT

The three metabolites of dipyrone found in plasma (4-methylaminoantipyrine, 4-aminoantipyrine and 4-formylaminoantipyrine) were separated and quantified by micellar liquid chromatography. The method used an 0.1 M sodium dodecyl sulphate mobile phase and an octadecyl silica bonded stationary phase. Dipyrone metabolites were measured by UV detection at 262 nm. The proposed method needs only 5 min to separate the three metabolites at a flow rate of 1 ml/min. The limits of detection (LODs) in plasma ranged between 0.1 and 0.2 ng injected. The method was applied to the determination of dipyrone metabolites in human plasma samples.

INTRODUCTION

Dipyrone [sodium N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methyl-amino methane sulphonate monohydrate] is an effective analgesic, antipyretic and anti-inflammatory drug widely used for some 60 years. The drug is rapidly hydrolysed to 4-methyl-aminoantipyrine (MAA) before its absorption from the gastro-intestinal tract. After absorption three major metabolites have been found in plasma: 4-methylaminoantipyrine (MAA), 4-aminoantipyrine (AA), and 4-formylaminoantipyrine (FAA). Consequently, the study of clinical pharmacokinetics of dipyrone requires precise determination of the concentrations of MAA, AA, and FAA in human plasma. Analytical

methods for the determination of dipyrone metabolites include reverse-phase high performance liquid chromatography (HPLC) (1,2) and thin layer chromatography (TLC) (3). Four dipyrone metabolites can be separated by HPLC, but the analysis requires at least 30 min of elution (1). The determination of only two metabolites, MAA and AA, can be carried out in 12 min (2). However, for pharmacokinetic studies in which a large

The use of micellar mobile phases for reverse-phase separations in high performance liquid chromatography was introduced by Armstrong and Henry in 1980[4]. Micellar liquid chromatography (MLC) offers important advantages of interest in analytical separations, including low toxicity, inflammability and cost of the mobile phase, the possibility of simultaneous elution of both hydrophilic and hydrophobic substances[5-7] and the ability to perform direct injection of biological samples into the chromatographic system without previous sample extraction (8-10). The main drawback of MLC is a large loss of efficiency when compared to conventional hydroorganic mobile phases. Dorsey et al. (11) explained that the low efficiency was due to poor mass transfer on bonded stationary phases as a result of the high water content of the micellar eluent. They showed that the addition of minute amounts of n-propanol to the micellar mobile phase and raising column temperature greatly improved the efficiency for non ionic solutes.

In the present paper, a method based on micellar liquid chromatography is proposed for the separation and quantitation of dipyrone metabolites in human plasma. The effects of SDS concentration and n-pentanol in the mobile phase are studied. Experimental conditions are selected to achieve separation in the shortest time. The method applicability for plasma analysis from a human volunteer is presented.

EXPERIMENTAL

Apparatus

The chromatographic separation was carried out at room temperature with a high-resolution liquid chromatograph consisting of a Spectra-Physics Isochrom LC pump, a Spectra 100 UV-VIS detector with wavelength fixed at 262 nm and a Spectra-Physics Chromjet integrator. The separation column was a Nucleosil-120-5-C_{18}, with 5-μm particle size, 25-cm length and 4-mm internal diameter. The mobile phase was 0.1 M SDS containing 2.5% (v/v) n-pentanol. The mixture was injected through a Rheodyne model 7010 injection valve with a 10 μl loop.

Reagents and Procedure

Both sodium dodecyl sulphate (SDS, Merck) and n-pentanol (Merck) were analytical reagent grade. The mobile phase was filtered through a Nylon-66 47-mm diameter membrane with 0.45 μm pore size and degassed under vacuum in an ultrasonic bath prior to use. The internal standard was furosemide (Sigma). The metabolites 4-methylaminoantipyrine (MAA), 4-aminoantipyrine (AA) and 4-formylaminoantipyrine (FAA) were a generous gift of Hoechst. All the compounds were used without further purification. Stock solutions used for calibration were prepared containing known amounts of the metabolites. The concentration ranged between 0.5 and 10 μg/ml with 10 μg/ml furosemide as internal standard.

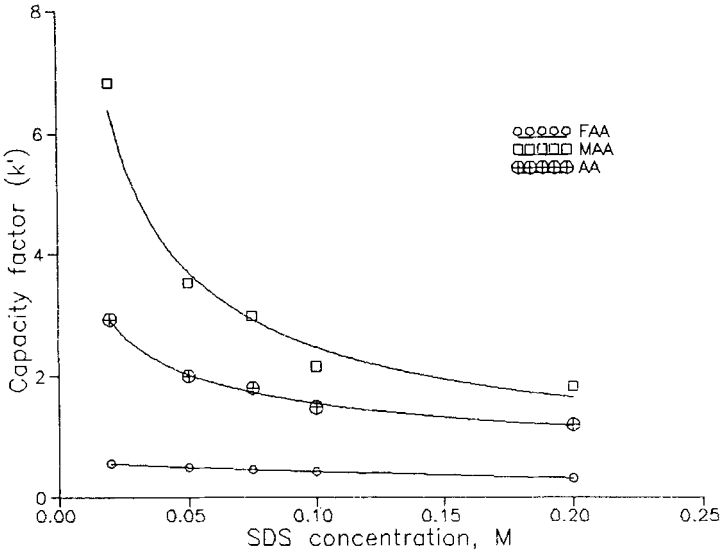

FIGURE 1: Variation of capacity factor (k') with concentration of surfactant in the mobile phase. Mobile phase: SDS aqueous solution containing 2 % pentanol.

Extraction Procedure

To 1 ml of plasma sample, 0.1 ml of standard solution containing the metabolites (MAA, AA, FAA, 40 μg/ml), 8 μl of 1 M sodium hydroxide and 5 ml of methylene chloride were added. The contents of the tubes were mixed for 1 min on a vortex-mixer and then centrifuged for 5 min at 2000 rpm. The aqueous phase was re-extracted with another 5 ml portion of methylene chloride following the same procedure. Organic phases of the two successive extractions were mixed and evaporated to dryness in a 40 °C water bath under a stream of air. The residue was reconstituted in 50 μl of mobile phase. A 10 μl aliquot of the extract was injected into the chromatographic system.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of SDS concentration in the mobile phase on retention for the three dipyrone metabolites. As the SDS concentration increases, k' values fall. This fact indicates that interactions in the mobile phase are no longer negligible, compared to interactions between the adsorbed ionic surfactant and the solutes. Armstrong and Stine[13] proposed a classification of the solutes according to their chromatographic properties in MLC solutes binding to micelles, non binding solutes and antibinding solutes.

Table I

Efficiency in theoretical plates (N) for various dipyrone metabolites as a function of SDS concentration in the mobile phase, containing 2 % pentanol.

	SDS concentration (M)				
Compound	0.02	0.05	0.75	0.1	0.2
FAA	1392	1579	1670	1970	1617
AA	838	705	791	1550	765
MAA	105	192	168	436	321

A 25 cm Nucleosil C_{18} column (5 μm) was used at a flow rate of 1 ml/min and at room temperature. Efficiencies were calculated by using $N = 41.7(t_r/W_{0.1})^2/(B/A + 1.25)$. t_r, retention time; $W_{0.1}$, peak width at 10% peak height; B/A, asymmetry factor;

Fig. 1 shows that retention of FAA was not appreciably modified by varying the SDS concentration and should be considered as a non-binding solute. On the other hand retention of MAA and AA decreases as the SDS concentration increases. Thus these solutes should be considered as binding solutes. SDS concentrations above 0.2 M result in a rapid elution of three metabolites.

Unfortunately, the low efficiency of MLC causes overlapping peaks at SDS concentrations 0.1 M. The efficiency variation using a mobile phase containing 2% pentanol and different SDS concentrations is illustrated in Table I. It is interesting to note that the three metabolites showed initial increases of N when increasing SDS concentration, to reach a maximum value at 0.1 M SDS. Further increase of SDS concentration decreased the N values as expected. The high SDS concentration in the mobile phase causes a decrease in N due to the lower diffusion coefficients of the solutes. Moreover, the number of surfactant molecules adsorbed onto the stationary phase would increase. Thus, as the surfactant is an anionic compound, the hydratation layer would prevent hydrophobic solutes to reach the stationary phase resulting in decreased mass transfer and efficiency (13). The increase in efficiency for SDS concentrations up to 0.1 M is due to the presence 2% pentanol in the mobile phase which improved mass transfer and counterbalanced the effect of adsorbed SDS.

The problem of low efficiency in MLC is solved today. Several authors studied the improvement in efficiency and the possible causes. Addition of short-chained alcohols reduces the number of surfactant molecules adsorbed on the stationary phase and changes the polarity of mobile phase, resulting in increased efficiency. The results for the dipyrone metabolites are summarized in Table II. The largest improvement in efficiency occurs for the metabolite that is most retained in the column, i.e., MAA. High pentanol concentrations cause high efficiencies but the selectivity of the method drops dramatically.

Table II

Efficiency in theoretical plates (N) for various dipyrone metabolites as a function of n-pentanol content of the 0.1 M SDS mobile phase. Experimental conditions as in Table I

	Pentanol concentration (%)						
Compound	0	0.5	1.5	2.0	2.5	4.0	7.1
FAA	276	1066	1086	1970	1267	2672	4995
AA	82	493	646	436	1506	3222	3319
MAA	31	380	895	550	262	454	3027

Fig. 2 shows the k' variation with pentanol concentration. For MAA the decrease in retention is quite significant, k' values decreasing from 40.21 min. for 0.1 M SDS without pentanol to 5.12 min for a mobile phase containing 7.1 % pentanol. This effect was less evident for FAA, where the values ranged between 6.94 min. and 2.46 min. For pentanol contents higher than 3% the three metabolites coelute. The best conditions for separation and quantitation corresponded to a mobile phase containing 0.1 M SDS with 2.5 % pentanol. Fig. 3 shows the chromatogram of a plasma sample spiked with the three metabolites and further extracted and analyzed by the method recommended. Furosemide is used as internal standard. Although furosemide and the three metabolites are not structurally similar, no reaction between them occurs, they elute separately, resulting in satisfactory analytical results. The metabolites are eluted in a short time period. The run time is 5 min.

Table III summarizes the analytical figures of merit of the method proposed. Linearity limits were obtained by plotting peak height vs. metabolite concentration (5% deviation from linearity). Values ranged from 14.4 ppm for FAA to 18 ppm for AA. The calibration curves pass through the origin, with correlation coefficients between 0.9988 for MAA and 0.9999 for AA.

The detection limits of the UV analysis of the three metabolites in plasma were in the range 0.1-0.2 ng injected. The precision of the analytical procedure was studied by six repeated injections of the same plasma sample containing 5 μg/ml of the three metabolites. The internal standard was added prior to the extraction of the metabolites. Recovery studies were assayed by spiking plasma samples with known amounts of the three metabolites and following extraction by the method described above. The extraction reproducibility was studied. The results are shown in Table IV. The data represent mean recoveries obtained in three consecutive days using furosemide as the internal standard. Relative standard deviations of the recovery ranged between 4.6 % for FAA and 7.1 % for MAA. The recovery ranged between 71.25% for FAA and 89.75 % for MAA. The poor recovery observed in some instances can be due to binding of the metabolites to plasma proteins.

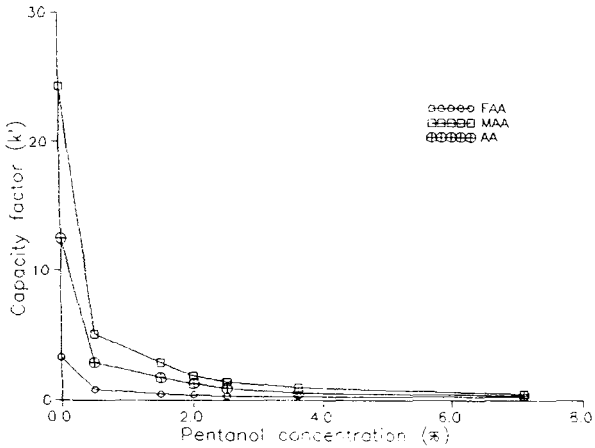

FIGURE 2: Variation of capacity factors (k') with concentration of pentanol in the mobile phase. Mobile phase: 0.1 M SDS aqueous solution containing different amounts of pentanol.

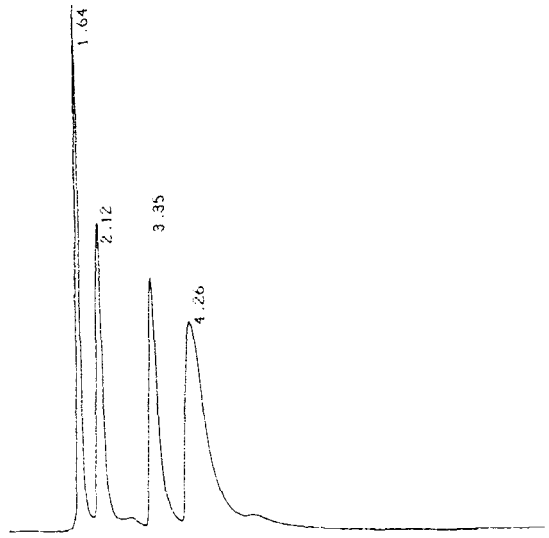

FIGURE 3: Chromatogram of plasma spiked with FAA (5 μg/ml, t_r = 2.12 min), AA (8 μg/ml, t_r = 3.35 min), MAA (8 μg/ml, t_r = 4.26 min) and furosemide (10 μg/ml, t_r = 1.64 min) was added prior to extraction at the aliquot injected. Mobile phase: 0.1 M SDS + 2.5 % pentanol. Column: Nucleosil C_{18} 25 x 0.4 cm, 5 μm. Operating at room temperature

Table III

Analytical figures of merit of the analytical procedure for dipyrone metabolites

Compound	Linearity limit (μg/ml)	LOD[a] (pg/ml)	r	s (μg/ml)	G (%)	RSD (%)
FAA	14.4	10.5 (0.1)	0.9998	0.07	1.44	1.8
AA	18.0	11.5 (0.1)	0.9999	0.11	2.33	2.2
MAA	16.4	17.0 (0.2)	0.9988	0.24	5.01	4.7

[a] In parenthesis, absolute amount injected in ng. Precision of the analytical procedure was calculated by repeated injection of a plasma sample containing the metabolites (5 μg/ml each) and the internal standard (10 μg/ml). r = correlation coefficient. $G = 100ts/x_m n$; x_m = mean value; n = number of determinations, in this case n=6; s = standard deviation; G (%) = mean relative error; t = Student's t for 95% confidence.

Table IV

Extraction reproducibility and mean recoveries of dipyrone metabolites in human plasma

Compound	Amount added (μg/ml)	Amount found[a](μg/ml)	RSD[b] (%)	Average recovery (%)
FAA	4.00	2.85	4.60	71.25
AA	4.00	3.11	6.80	77.75
MAA	4.00	3.59	7.10	89.75

[a] Data were obtained of value mean recoveries in three consecutive days. Samples were assayed by quintuplicate, using furosemide as internal standard. [b] Relative standard deviation of the recovery.

Table V

Plasma concentration (μg/ml) of dipyrone metabolites following intravenous injection of 2.0 g dipyrone to a healthy volunteer

	Extraction time after dosis, min					
Compound	10	30	50	80	170	290
FAA	0.5	1.2	2.1	2.5	2.7	4.8
AA	2.1	1.9	2.7	3.2	4.2	4.9
MAA	62.4	29.6	28.7	16.9	15.9	10.7

Human Plasma Analysis

Plasma samples were extracted from a healthy volunteer which received 2 g dipyrone by intravenous injection and further analyzed with the method proposed. Table V summarizes the pharmacokinetics of the three dipyrone metabolites. The MAA level is high early after injection, to decrease afterwards. The plasma concentrations of AA and FAA increase slowly, to reach balanced concentrations after 5 hr from dosis. These results suggest that dipyrone is first metabolized to MAA. Further metabolism results in AA and FAA. This metabolic pattern is in agreement with previous pharmacokinetics studies of dipyrone (14), indicating the suitability of the proposed method for this purpose.

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