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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS FOR THE ANALYSIS OF ANALGESIC AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN FORENSIC TOXICOLOGY

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SUMMARY

High-performance liquid chromatography retention data are presented for over 40 analgesic drugs on an ODS-silica packing material to assist in the identification of these compounds. Three isocratic eluents prepared from isopropanol, formic acid and an aqueous phosphate buffer have been used. One eluent has been used for the analysis of paracetamol in whole blood.

INTRODUCTION

Within the last decade the number of analgesic and non-steroidal anti-inflammatory drugs available to the public has increased markedly. This has enhanced the possibility of such drugs occurring in toxicological cases. Many of these compounds are polar, making high-performance liquid chromatography (HPLC) a particularly suitable technique for the purposes of identification and/or quantification.

The scientific literature contains many specific HPLC methods for the analysis of analgesic drugs in various matrices including pharmaceuticals and body fluids. A review of this general area has been published[1] while some detailed surveys for particular drugs have also appeared (*e.g.* salicylates[2], paracetamol[3], phenacetin[3]). Most papers present retention data for only a small number of analgesic drugs, and the available information covers a wide range of different packing materials and eluents. A few papers have presented retention data for groups of ten or more compounds[4,5] often when considering interferences with specific assays, but no attempt has been made to measure the retention properties of a large group of these drugs on a single packing material.

Most workers have used reversed-phase HPLC systems for the analysis of analgesic drugs. The forensic science laboratories in the U.K. have recently standardized on a particular brand of ODS-silica (ODS-Hypersil), to facilitate the rapid transfer of HPLC methods between laboratories. Current work involves the provision of a large data base of retention properties on this material. To date data have been published for barbiturates[6,7], amphetamines[8], cocaine and related local anaesthet-

ics[9], LSD and other ergot alkaloids[10] and benzodiazepines[11]. It was the intention of the present work to record the retention properties of a large group of analgesic drugs on ODS-Hypersil using suitable eluents.

EXPERIMENTAL

Apparatus

HPLC was carried out using a Waters M6000 pump, a Rheodyne 7125 injection valve (fitted with a 10-μl loop) and a Pye-Unicam LC-UV variable-wavelength detector operated at 240 nm. The stainless-steel column (16 cm × 5 mm I.D.) was packed with 5 μm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.) by a slurry procedure, dispersing the packing material in isopropanol with hexane as the pressurizing solvent.

Materials

Isopropanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Potassium dihydrogen orthophosphate (AnalaR), perchloric acid, 72% w/w (AristaR), and formic acid, 98–100% (AnalaR) were obtained from BDH (Poole, U.K.). All other chemicals used were of analytical grade.

Pure samples of paracetamol, its glucuronide, sulphate, cysteine, and mercapturic acid metabolites, and a pure sample of N-propionyl-*p*-aminophenol were obtained from the Sterling Winthrop Research Centre (Alnwick, U.K.). The remaining analgesic drugs were from the drug collection of the Central Research Establishment (Home Office Forensic Science Service, Aldermaston, U.K.).

Chromatography

Three eluents were required to elute the analgesic drugs. The eluents were prepared by mixing aqueous potassium dihydrogen phosphate (1000 ml, 0.1 *M*) with formic acid (1 ml) and isopropanol (x ml), where x = 17 ml, 176 ml and 540 ml for eluents 1, 2 and 3 respectively. The eluents had isopropanol contents of 1.7%, 15% and 35% respectively. All eluents were thoroughly degassed with helium before use. Flow-rates were 2 ml/min for eluent 1 and 1.5 ml/min for eluents 2 and 3. The column was allowed to equilibrate for one hour with each eluent before beginning retention measurements.

Drug samples were dissolved in a minimum volume of methanol or isopropanol and diluted with the appropriate eluent prior to injection on to the HPLC column. The paracetamol metabolites were injected in water.

Analysis of paracetamol in whole blood

The blood precipitant containing internal standard was prepared by diluting perchloric acid (72% w/w, 4 ml) with water (100 ml) and adding N-propionyl-*p*-aminophenol (5 mg).

Each blood sample was given gentle vortex mixing before analysis to ensure homogeneity and then 100 μl was transferred to a small glass test tube (50 × 6 mm). The blood precipitant containing internal standard was added and the liquid vortex mixed for 20–30 s. (The internal standard was added at a concentration equivalent to 100 μg/ml in the blood.) The mixture was centrifuged at 9000 *g* for approximately

45 s and the supernatant liquid decanted into a clean test tube where it was subjected to a further centrifugation (approximately 20 s). Aliquots (5 μl) of the clear supernatant were injected on to the HPLC system (eluent 1).

Quantification was performed using peak height ratio measurements (paracetamol–internal standard) with changes to the detector sensitivity where necessary. The calibration graph was prepared by conducting the assay with swine serum spiked with the drug.

RESULTS AND DISCUSSION

Initial experiments with various eluents on the ODS-Hypersil column revealed that the analgesic drugs show a very wide range of retention properties and that a series of isocratic eluents would be required. Paracetamol and its metabolites were found to be amongst the most polar compounds showing low retention on the column. As paracetamol is frequently encountered in forensic toxicology casework[12] it was considered desirable that one of the isocratic eluents selected should be appropriate for the analysis of this drug. An HPLC system published by Buchanan *et al.*[13] involving an eluent containing isopropanol–0.1 *M* potassium dihydrogen phosphate–formic acid (17:1000:1, v/v/v) was found to be particularly suitable and this was adopted as eluent 1. The experimental work confirmed that the parent drug was well resolved from its metabolites with good peak shapes (Fig. 1A). The capacity ratios of the compounds eluted on this system are given in Table I in order of increasing retention.

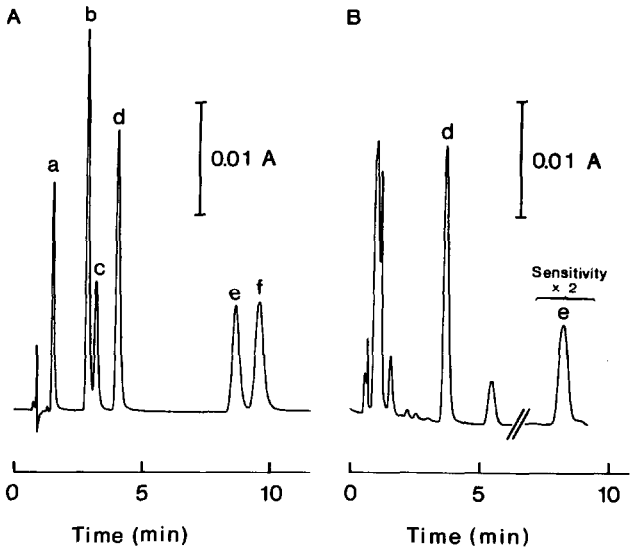

Fig. 1. Separation of paracetamol and its metabolites using eluent 1. (A) Standard mixture; (B) post mortem blood containing 300 μg/ml paracetamol. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 1.7% isopropanol containing a formic acid–phosphate buffer; flow-rate: 2 ml/min; detection: 240 nm. Peaks: a = paracetamol glucuronide; b = paracetamol sulphate; c = paracetamol cysteine; d = paracetamol; e = N-propionyl-*p*-aminophenol (internal standard for blood analysis); f = paracetamol mercapturide.

TABLE I

HPLC RETENTION DATA FOR PARACETAMOL AND ITS METABOLITES, IN ORDER OF INCREASING RETENTION, USING ELUENT 1

Eluent 1 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (17:1000:1, v/v/v).

Compound	*Capacity ratio (k')*
Paracetamol glucuronide	0.7
Paracetamol sulphate	2.1
Paracetamol cysteine	2.3
Paracetamol	3.2
N-propionyl-*p*-aminophenol*	7.9
Paracetamol mercapturide	8.6

* Internal standard used for the quantification of paracetamol in blood.

All the other analgesic drugs considered in the present study gave capacity ratios (k') greater tha 10 with eluent 1 except for amidopyrine which gave a broad tailing peak at the same retention time as paracetamol mercapturide. Two further isocratic eluents containing higher proportions of isopropanol were found to be necessary to elute the other compounds with satisfactory retention properties. These eluents were prepared from the same components as eluent 1 but with 15% and 35%

TABLE II

HPLC RETENTION DATA FOR ANALGESIC DRUGS, IN ORDER OF INCREASING RETENTION, USING ELUENT 2

Eluent 2 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (176:1000:1, v/v/v).

Compound	*Capacity ratio (k')*
Amidopyrine	0.32*
Paracetamol	0.32
Dipyrone	0.45*
Nifenazone	0.45*
Phenazone	0.95*
Morazone	2.05*
Acetanilide	2.3
Salicylamide	2.5
Acetylsalicylic acid	2.7
Phenacetin	4.4
Etenzamide	4.6
Salicylic acid	4.6
Choline salicylate	4.8
Glycol salicylate	7.3
Piroxicam	7.7
Propyphenazone	11.0
Benorylate	22.4

* Tailing peaks.

TABLE III

HPLC RETENTION DATA FOR ANALGESIC DRUGS, IN ORDER OF INCREASING RETENTION, USING ELUENT 3

Eluent 3 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (540:1000:1, v/v/v).

Compound	*Capacity ratio (k')*	*Compound*	*Capacity ratio (k')*
Dipyrone	0.1	Bufexamac	1.95
Nifenazone	0.1	Oxyphenbutazone	1.95
Paracetamol	0.1	Tolmetin	2.05
Phenazone	0.1	Ketoprofen	2.4
Amidopyrine	0.2	Famprofazone	2.5
Morazone	0.4	Alclofenac	2.6
Salicylamide	0.4	Naproxen	3.3
Acetanilide	0.5	Salsalate	3.6
Acetylsalicylic acid	0.5	Zomepirac	3.7
Etenzamide	0.55	Methyl salicylate	3.9
5-Hydroxyindoprofen	0.6	Fenbufen	4.0
Phenacetin	0.6	Diflunisal	4.1
Piroxicam	0.6	Phenylbutazone	6.5
Benorylate	0.7	Indomethacin	6.95
Choline salicylate	0.7	Fenoprofen	7.9
6-Hydroxyindoprofen	0.7	Benoxaprofen	11.3
Salicylic acid	0.7	Diclofenac	11.5
Glycol salicylate	1.0	Ibuprofen	15.1
Indoprofen	1.2	Phenyl salicylate	15.6
Propyphenazone	1.25	Flufenamic acid	19.7
Sulindac	1.25	Mefenamic acid	21.2

isopropanol for eluents 2 and 3 respectively. Capacity ratios for the drugs and metabolites with these two eluents are presented in Tables II and III in order of increasing retention. The complete collection of retention data is given in Table IV where the compounds are listed in alphabetical order allowing the rapid selection of the appropriate eluent for a given drug.

The selection of eluents 2 and 3 followed a series of experiments to examine the influence of isopropanol concentration on chromatographic separation. Fig. 2 shows the results for eight drugs selected to be representative of those having short to intermediate retention. Overall the effect is as expected, with k' values decreasing with increasing isopropanol concentration. Where data are available all compounds eluted in the same order at different isopropanol concentrations except for 5-hydroxyindoprofen, 6-hydroxyindoprofen and salicylic acid which show changes between 25% and 30% organic modifier and for sulindac and oxyphenbutazone in the same region.

Generally all the compounds examined using eluents 2 and 3 gave satisfactory peak shapes except for amidopyrine, dipyrone, morazone, nifenazone and phenazone which showed slight tailing. Fig. 3 shows the separation of a standard mixture of six compounds using eluent 2 while the separation of a standard mixture of twelve analgesics using eluent 3 is shown in Fig. 4.

TABLE IV

HPLC RETENTION DATA FOR ANALGESIC DRUGS, ARRANGED IN ALPHABETICAL ORDER

Eluents: isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (x : 1000:1, v/v/v), x = 17, 176 and 540 ml for eluents 1, 2 and 3 respectively.

Compound	*Capacity ratio (k')*		
	Eluent 1	*Eluent 2*	*Eluent 3*
Acetanilide		2.3	0.5
Acetylsalicylic acid		2.7	0.5
Alclofenac			2.6
Amidopyrine	8.6	0.32	0.2
Benorylate		22.4	0.7
Benoxaprofen			11.3
Bufexamac			1.95
Choline salicylate		4.8	0.7
Diclofenac			11.5
Diflunisal			4.1
Dipyrone		0.45	0.1
Etenzamide		4.6	0.55
Famprofazone			2.5
Fenbufen			4.0
Fenoprofen			7.9
Flufenamic acid			19.7
Glycol salicylate		7.3	1.0
5-Hydroxyindoprofen			0.6
6-Hydroxyindoprofen			0.7
Ibuprofen			15.1
Indomethacin			6.95
Indoprofen			1.2
Ketoprofen			2.4
Mefenamic acid			21.2
Methyl salicylate			3.9
Morazone		2.05	0.4
Naproxen			3.3
Nifenazone		0.45	0.1
N-propionyl-*p*-aminophenol*	7.9		
Oxyphenbutazone			1.95
Paracetamol	3.2	0.32	0.1
Paracetamol cysteine	2.3		
Paracetamol glucuronide	0.7		
Paracetamol mercapturide	8.6		
Paracetamol sulphate	2.1		
Phenacetin		4.4	0.6
Phenazone		0.95	0.1
Phenylbutazone			6.5
Phenylsalicylate			15.6
Piroxicam		7.7	0.6
Propyphenazone		11.0	1.25
Salicylamide	14.0	2.5	0.4
Salicylic acid	21.6	4.6	0.7
Salsalate			3.6
Sulindac			1.25
Tolmetin			2.05
Zomepirac			3.7

* Internal standard used for the quantification of paracetamol in blood.

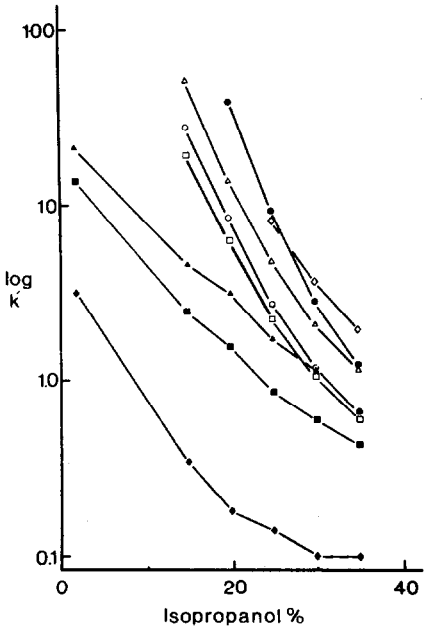

Fig. 2. The effect of isopropanol concentration on the retention of various analgesic drugs. Eluents: x% isopropanol containing a formic acid–phosphate buffer. Drugs: ◆ = paracetamol; ■ = salicylamide; ▲ = salicylic acid; ● = sulindac; ◇ = oxyphenbutazone; □ = 5-hydroxyindoprofen; ○ = 6-hydroxyindoprofen; △ = indoprofen.

Analysis of paracetamol in whole blood

The work of Buchanan *et al.*[13] describing one of the HPLC eluents adopted in the present study also describes a rapid sample preparation procedure for the quantification of paracetamol in human plasma. No information was presented in the original paper on the applicability of the method to the type of blood sample

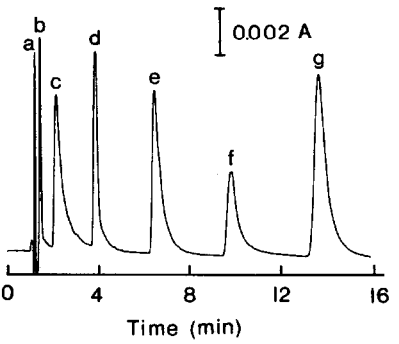

Fig. 3. Separation of analgesic drugs using eluent 2. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 15% isopropanol containing a formic acid–phosphate buffer; flow-rate: 1.5 ml/min; detection: 240 nm. Peaks: a = solvent disturbance; b = paracetamol; c = phenazone; d = salicylamide; e = salicylic acid; f = piroxicam; g = propyphenazone.

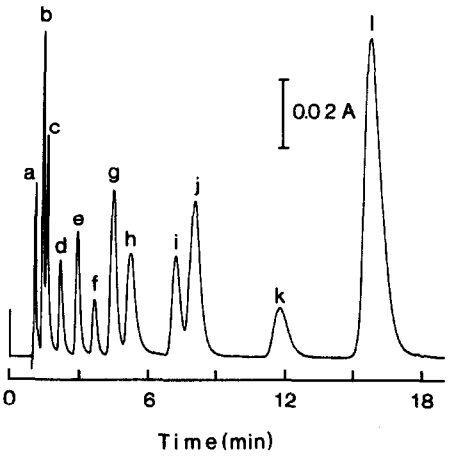

Fig. 4. Separation of analgesic drugs using eluent 3. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 35% isopropanol containing a formic acid–phosphate buffer; flow-rate: 1.5 ml/min; detection: 240 nm. Peaks: a = paracetamol; b = salicylamide; c = phenacetin; d = propyphenazone; e = oxyphenbutazone; f = alclofenac; g = salsalate; h = diflunisal; i = phenylbutazone; j = indomethacin; k = diclofenac; l = phenylsalicylate.

likely to be encountered in forensic toxicology where the isolation of plasma is often impossible because of haemolysis and/or putrefaction[14]. As an extension of the present study the applicability of eluent 1 for the analysis of paracetamol in whole blood using the approach of Buchanan *et al.*[13] has been examined.

The published procedure involved the addition of perchloric acid (0.33 *M*, 100 μl) to plasma (100 μl) causing the precipitation of protein which was removed by centrifugation at 9000 *g* before injection of the supernatant on to the HPLC column. Experiments showed that the replacement of plasma by whole blood gave incomplete precipitation and the method required minor modification involving an increase in the volume (200 μl) and concentration (0.49 *M*) of the perchloric acid. The modified method has been successfully tested on a wide range of different whole blood samples including specimens provided for the investigation of road traffic offences and also putrefying post mortem material. Fig. 1B shows a typical chromatogram arising from a fatal case involving paracetamol overdose. Interferences with the assay by endogenous compounds have not been encountered and the retention data presented in this paper for other analgesics indicate that no interferences occur from these drugs.

Quantification was performed by peak height ratio measurements of paracetamol relative to the internal standard (N-propionyl-*p*-aminophenol) which was added with the perchloric acid. Calibration graphs were constructed by conducting the assay with spiked swine serum samples. Using the assay procedure with external calibration, the recoveries of paracetamol added to water, swine serum and whole blood were measured as 97.2%, 102.7% and 91.7% respectively at 10 μg/ml and 102.5%, 105.1% and 88.0% respectively at 200 μg/ml. Similarly recoveries of the internal standard (100 μg/ml) were found to be 100.0%, 100.0% and 82.7% respectively. A linear relationship between the paracetamol–internal standard peak height ratio and the concentration of paracetamol in blood up to 250 μg/ml was demon-

strated for the method. In a collaborative study between 11 U.K. forensic science laboratories two samples of blood were spiked with paracetamol at 13.0 μg/ml and 235 μg/ml respectively. The results showed mean values of 13.3 μg/ml and 238.7 μg/ml with coefficients of variation of 16.8% and 5.61% respectively.

CONCLUSIONS

The present paper presents a series of three isocratic eluents on a single HPLC column (ODS-Hypersil) which are suitable for the analysis of a wide range of analgesic, anti-inflammatory drugs and should prove useful for the identification and quantification of these drugs in forensic science laboratories. A rapid sample preparation procedure for paracetamol analysis in whole blood has also been examined which can be used with one of the HPLC eluents. Similar protein precipitation procedures have been used for the analysis of other analgesic drugs in blood when concentrations are relatively high (*e.g.* aspirin and metabolites[15,16] and diflunisal[15]. However, experience has shown that some analgesic drugs give poor recoveries with such procedures and further work is required to explore alternative sample preparation procedures to facilitate the full exploitation of these HPLC systems for blood analysis.

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