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Liquid chromatographic–thermospray tandem mass spectrometric quantitative analysis of some drugs with hypnotic, sedative and tranquillising properties in whole blood

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Abstract

Results are presented of a liquid chromatographic–thermospray tandem mass spectrometric method of analysing different drugs in whole blood. Substances with hypnotic, sedative and tranquillising properties from the benzodiazepine, the thioxanthene, the butyrophenone, the methadone and the diphenylbutylpiperidine groups were investigated. It appears that ten to hundred times lower detection limits for the substances in whole blood can be reached with this method compared with methods more commonly used. Detection limits in the range 10–100 pg per injection (equivalent to 0.05–0.5 ng/ml whole blood) were reached for the majority of the compounds

Keywords: Benzodiazepines; Thioxanthenes; Butyrophenones; Methadones; Diphenylbutylpiperidines

1. Introduction

In toxicology great importance is attached to lowering the limits of detection of drugs present in body fluids or other body specimens. This is not only for answering questions about therapeutic, toxic or lethal amounts of administered drugs or poisons, but also for analytical checking of the clinical history of a deceased or the statements made by witnesses during the course of a police inquiry [1].

A number of methods are in use for qualitative and quantitative analysis of drugs in body specimens. Well known are immunoassays as radioimmunoassay and several optical immunoassays [2]. Furthermore, gas chromatography (GC) can be used with various detection systems like flame-ionization detection (FID), nitrogen–phosphorus detection (NPD) or electron-capture detection (ECD) [3] and GC–mass spectrometry (MS) with the different ionization modes [electron impact (EI) and chemical ionization (CI)] or scanning modes [full scan, selected-ion monitoring (SIM) or multiple-ion detection (MID)] [4]. A method of increasing importance for these purposes is high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence or electrochemical detection [5] and even LC combined with MS can be used [6].

Due to the complex nature of urine, blood or other biological matrices most procedures for extraction and isolation of drugs yield samples with different degrees of purity. For that reason the analytical methods mentioned are frequently hampered by impurities which can cause severe inconvenience in the quantification process of the drugs. Therefore specific analyses should be applied in order to obtain reliable analytical results.

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One of the methods with a high degree of specificity is tandem mass spectrometry (MS–MS), where selectivity is provided by mass separation in the first mass analyzer. Thus MS–MS can be used for selective separation and identification of compounds with the same retention time but of differing molecular mass. Further specificity and increased sensitivity (by noise suppression) can be obtained by applying collision-induced dissociation of the preselected precursor ion, to be transferred by the first mass analyzer, generating in that way a characteristic set of ions, to be analysed in the second mass analyzer (daughter ion scan) [4,6–9]. Collision-induced dissociation spectra can uniquely identify a particular compound and this technique can be used to distinguish compounds with the same molecular mass.

We present here the results of a study of the possibilities of the use of LC with a thermospray (TSP) ionization interface in conjunction with a mass spectrometer with MS–MS capabilities for the analysis of drugs of the benzodiazepine, thioxanthene, methadone, butyrophenone and diphenylbutylpiperidine groups in whole blood. The mass spectrometer was used in the daughter-ion scan mode and optimum selectivity was obtained by operating the MS–MS under the so called selected reaction monitoring (SRM) technique [4,6–8]. Because of thermolability of some of the studied compounds LC instead of gas chromatography was chosen as separation method.

The results obtained in this way will be compared with other, more conventional methods for analysing these types of drugs.

2. Experimental

2.1. Chemicals

The benzodiazepines studied were brotizolam, clonazepam, desmethylflunitrazepam, diazepam, flunitrazepam, ketazolam, loprazolam, lormetazepam, nitrazepam and triazolam; the thioxanthenes studied were chlorprothixene, flupenthixol, thiothixene and clopenthixol; the members of the methadone group studied were dextromoramide, dextropropoxyphene and methadone; the butyrophenones studied were benperidol, droperidol, haloperidol and pipamperone; the diphenylbutylpiperidines studied were penfluridol and pimozide. The compounds were donated as pure substances by the producers or their representatives: Boehringer, BUFA, Hoffmann-La Roche, Janssen, Lundbeck, Parke Davis, Pfizer, Roussel, Upjohn and Wyeth.

Blood was outdated transfusion blood and was frozen until used. Water was purified by the Milli Q–Organex System (Millipore, Milford, MA, USA). Acetonitrile, chloroform, dichloromethane, diethyl ether and methanol were of HPLC and glass-distilled grade (Rathburn, Walkerburn, UK). All other reagents were of analytical grade. Extractions were done with Bond Elut Certify columns (Varian, Walnut Creek, CA, USA)

2.2. Equipment

A Waters (Milford, MA, USA) 600-MS programmable pump equipped with a U6K injector was used for HPLC. The following columns and flows gave good separations between the different drugs of a group.

For benzodiazepines, a Waters 4-μm Nova-Pak C_{18} column, 150×3.9 mm I.D. was used with a mobile phase of methanol–50 m*M* ammonium acetate in water (60:40, v/v) at a flow-rate of 0.4 ml/min.

For thioxanthenes, a Hewlett-Packard (Palo Alto, CA, USA) 5-μm Asahipak OPD-50 column, 125×4.0 mm I.D. was used with a mobile phase of acetonitrile–50 m*M* ammonium acetate in water (85:15, v/v) at a flow-rate of 0.6 ml/min.

For butyrophenones, methadones and diphenylbutylpiperidines, a Waters 4-μm Nova-Pak C_{18} column, 150×3.9 mm I.D. was used with a mobile phase of methanol–50 m*M* ammonium acetate in water (75:25, v/v) at a flow-rate of 0.6 ml/min.

In all cases an extra 0.8 ml/min of 50 m*M* ammonium acetate was added post column by a Waters 590-MS isocratic pump for ionizing enhancement in thermospray applications.

A Finnigan MAT TSQ 700 triple quadrupole mass spectrometer coupled to a DEC station 2100 was used. The liquid chromatograph was connected to the mass spectrometer by a Finnigan MAT TSP-2 inter-

face. The operating conditions of the interface, including the repeller voltage, vaporizer temperature, source temperature, and ionic strength of the eluent, were all optimized for the different compounds (see Tables 1–3).

MS–MS experiments in the daughter-ion mode were performed with the triple-stage quadrupole mass spectrometer (Q1, Q2, Q3). In these MS–MS experiments, the $[M+H]^+$ quasi molecular ion was chosen as the precursor ion and selectively transmitted by Q1 for further collisional dissociation in Q2 [6–8]. Argon was used as the collision gas and the pressure was between 200 and 530 mPa (see Tables 1–3). Varying collision offset voltages were applied to Q2. The collision-activated dissociation (CAD) daughter ions thus obtained were then analyzed by scanning with the third quadrupole (Q3) over the mass range m/z 40–600 in full scan mode. In order to obtain optimum selectivity for the different drugs, the SRM technique, and not the full-scan technique, was applied [6–8]. In this case, only one special ion is allowed to pass the third quadrupole (Q3). Selectivity is then extraordinarily increased, by almost completely suppressing the noise level. Collision offset voltage, argon pressure, and MSMSC factor (a correction factor for transmission of ions in the MS–MS mode) were all optimized, and the most intense fragment ion in the MS–MS daughter spectrum was chosen for the SRM experiments (see Tables 1–3).

2.3. Reference solutions

Stock solutions of the different substances were prepared by dissolving 10 mg of the pure substance in 10 ml of methanol. From these, diluted solutions of 1, 10, 100, 1000 ng/ml of the various compounds were prepared by addition of methanol. A 10-μl volume was injected. All reference solutions were stored in glass vials with PTFE-coated silicone rubber lined crimp caps. Whole blood was spiked by adding a quantity of the drug in methanol, taking care that the amount of methanol did not exceed 2%. Blood was spiked with flunitrazepam, desmethylflunitrazepam, lormetazepam, chlorprothixene, dextromoramide and haloperidol in concentration levels of approximately 10, 20, 50, 100 and 250 ng/ml each.

2.4. Sample treatment

The extraction procedure for the drugs from whole blood was the standard one we use in our laboratory for routine determinations of a general unknown in blood by HPLC with UV detection. Solid-phase extractions were performed with Bond Elut Certify 3-ml columns (Varian). Preconditioning of the column was done with 2 ml of 0.1 *M* phosphate buffer, pH 6.0. Care was given to wetting the column until the prepared blood sample was brought on the column. The preparation of the blood sample was accomplished by diluting 1 ml of blood with 6 ml of 0.1 *M* phosphate buffer, pH 6.0, in a polypropylene tube. After vortex-mixing and sonification, the solution was centrifuged, and the clear solution was transferred to the column. The column was then rinsed with water, followed by 1 m*M* acetic acid (pH 3.3). Afterwards, the column was dried by suction. Elution was done first with 2 ml of acetone–chloroform (50:50), giving an acidic fraction, followed by elution with 3 ml of a freshly prepared ethyl acetate–ammonia solution (98:2), giving the neutral and the basic fraction which was used in our experiments. The solvent of the latter fraction was evaporated at 40°C under a slight stream of nitrogen. The extract was dissolved in 50 μl of methanol, and an aliquot of this solution (10 μl) was injected into the chromatographic system.

3. Results and discussion

The TSP and MS–MS parameters are given in Table 1, Table 2 and Table 3 for the various compounds. In optimizing the thermospray parameters we found that moderately low repeller voltages could be used in all experiments and that a rather high vaporizer temperature was beneficial in terms of signal-to-noise ratio. Application of lower temperatures of the vaporizer gave less noise, but also far less signal. Variations of the collision gas pressure are of relatively little importance regarding sensitivity of the SRM method, but the values of the collision offset voltages and the MSMSC factor are of utmost importance. Slight variations of these parameters give undesirably large changes in sensitivity.

The detection limits in the SRM mode are with the

Table 1
HPLC–MS–MS parameters and detection limits for reference solutions of drugs of the benzodiazepine group[a]

Drug conditions[b]	MS	Collision offset (V)	Retention time(s)	Detection limit on column (pg)
Brotizolam	395–314	−30	563	50
Clonazepam	316–151	−60	373	20
Desmethylflunitrazepam	300–198	−40	333	20
Diazepam	285–165	−50	927	70
Flunitrazepam	313–268	−25	396	20
Ketazolam	285–165	−50	939[c]+1112	200
Loprazolam	465–408	−30	273	100
Lormetazepam	335–287	−20	634	10
Nitrazepam	282–152	−60	388	10
Triazolam	343–308	−33	493	70

[a] Using the SRM technique (signal-to-noise ratio approximately equal to 3). TSP-MS conditions: repeller, 70 V; vaporizer temperature, 140°C; source temperature, 200°C; MSMSC, 0; P_{Argon}, 530 mPa; multiplier voltage 1800 V; dynode power, 15 kV; Scan time, 1, 2 s.
[b] Quasi molecular ion (parent ion) and preferentely available daughter ion under the described conditions.
[c] Ketazolam decomposes to diazepam in aqueous solutions.

Table 2
HPLC–MS–MS parameters and detection limits for reference solutions of drugs of the thioxanthene group[a]

Drug conditions[b]	MS	Collision offset (V)	Retention time (s)	Detection limit on column (pg)
Chlorprothixene	316–271	−17.5	270	100
Flupentixol	435–265	−35	195	2000
Thiothixene	444–335	−15	150	5000
Clopenthixol	401–128	−17.5	220	2000

[a] Using the SRM technique (signal-to-noise ratio approximately equal to 3). TSP-MS conditions: repeller, 70 V; vaporizer temperature, 130–135°C; source temperature, 200°C; filament off; MSMSC, 0; P_{Argon} 330–530 mPa; multiplier voltage, 1800 V; dynode power, 15 kV; filament off; scan time, 1, 2 s.
[b] See Table 1.

Table 3
HPLC–MS–MS parameters and detection limits for reference solutions of drugs of the methadone, butyrophenone and diphenylbutylpiperidine groups[a]

Drug conditions[b]	MS	Collision offset (V)	Retention time (s)	Detection limit on column (pg)
Dextromoramide	393–306	−20	530	50
Dextropropoxyphene	340–266	−6	430	50
Methadone	310–265	−10	465	50
Penfluridol	524–109	−40	830	100
Pimozide	462–328	−27.5	620	100
Benperidol	382–165	−20	220	10
Droperidol	380–165	−25	250	10
Haloperidol	376–165	−27	230	50
Pipamperone	376–165	−25	230	100

[a] Using the SRM technique (signal-to-noise ratio approximately equal to 3). TSP-MS conditions: repeller, 100 V; vaporizer temperature, 130°C; source temperature, 200°C; filament on; MSMSC 10; P_{Argon}, 330 mPa; multiplier voltage, 1500 V; dynode power, 15 kV; scan time, 1, 2 s.
[b] See Table 1.

exception of the members of the thioxanthene group 20 to 100 times better than in the full-scan mode. The sensitivity of the SRM mode for the thioxanthenes was higher (four times) or lower (up to five times) than the sensitivity found in the full scan mode (depending upon the compound investigated). Here, as in all cases, the sensitivity in the SRM mode is influenced to a moderate degree by the presence of other ions in Q1, in addition to the nearly always dominating protonated molecular ion in Q1. Further, the detection limits in the SRM mode can be influenced by the fact that although the parameters regulating the collision activation process (in Q2) are chosen with utmost care, the ideal collision activation process for analytical quantitive purposes in which only one dissociation product is produced, in equal amounts, from the starting product $[M+H]^+$, is seldom met. Depending on these two interacting processes the detection limits in the full-scan and the SRM mode for the different compounds can vary. In the case of the thioxanthenes we have, in our opinion, a lowering of sensitivity in the SRM mode caused by an excessive collision activation process, giving a variety of degradation products of low yield.

All the preferential m/z values used in the SRM analytical procedure for the different drugs are given in the Tables 1–3 and were tested for the possible, disturbing presence of the same ions originating from blood extracts. No such interferences were found.

For whole blood the detection limits found for the described LC–MS–MS method for the different members of the groups studied appeared to be ten to a hundred times better than the levels found in literature. This was found for the radio immunoassay method [10,11], but particularly for the GC–FID [12], NPD [13,14] methods and the LC–UV [15–21] method. In several cases the LC–MS–MS method surpassed the limits given here.

In forensic toxicology mostly postmortem blood samples are available. Thus we were forced to develop a method as independent as possible of impurities in the blood extract. In literature (nearly always GC or HPLC) methods can be found for the analysis of most of the compounds studied here. There, a preference is found for the use of serum or plasma matrices, giving cleaner extracts as compared to whole blood. The given sensitivities of these methods of analysis [22–40] using serum or plasma extracts are of the same order as the sensitivities found in this study for the LC–MS–MS method using whole blood extracts.

We checked the extraction method used in this laboratory in cases of a general unknown in blood, as described in sample treatment. For this purpose we used the representative compounds desmethylflunitrazepam, flunitrazepam, lormetazepam, chlorprothixene, haloperidol and dextromoramide, because the other substances mentioned in this study can be thought of having nearly the same extraction properties. We analyzed at least four reference solutions of different concentrations of the compound in duplicate and at least four extracts of whole blood spiked with different amounts of these compounds in duplicate. As each extraction was done in duplicate four calibration graphs were obtained. Correlation coefficients, intercepts and slopes are given in Table 4. From the slopes found for the reference solutions and the spiked blood samples, the extraction yields could be calculated. Recoveries between 50% (benzodiazepines) and 90% (some compounds of the other groups) were found, but the low recovery of

Table 4
Statistical data for the calibration graphs

Compound	Slope (counts×μl×10^4/pg)	Intercept (counts×10^4)	r^2
Desmethylflunitrazepam			
Standard	2.81	0.11	0.99
Blood	1.68	2.16	0.98
Flunitrazepam			
Standard	2.13	0.07	0.99
Blood	1.48	11.17	0.95
Lormetazepam			
Standard	1.16	0.15	0.99
Blood	0.92	1.51	0.97
Chlorprothixene			
Standard	0.24	−2.12	0.98
Blood	0.25	1.01	0.99
Dextromoramide			
Standard	3.04	5.38	0.99
Blood	2.79	−5.13	0.99
Haloperidol			
Standard	0.85	9.73	0.99
Blood	0.71	−5.22	0.96

the benzodiazepines is compensated by the excellent sensitivity found for this group of compounds.

Overall standard deviation was estimated for spiked blood samples of some compounds. At a concentration level of 10 ng/ml the standard deviation was found to be between 5 and 10% ($n=6$).

As an example, a typical calibration curve is given in Fig. 1, which was used in forensic casework in the explanation of a sudden death.

In Fig. 2 a chromatogram is given showing the separation of haloperidol and dextromoramide.

The LC–MS–MS technique described here for the quantitative analysis of the substances mentioned, is a typical target compound method. Starting point is the presupposed presence of the compound to be analysed, which is a fundamental weakness of the method. In principle this weakness is also met in GC–MS–SIM methods, even when deuterated internal standards are used.

4. Conclusion

The LC–TSP-MS–MS–SRM method in the daughter-ion scan mode can be successfully applied to some members of the benzodiazepine, the thioxanthene, the methadone, the butyrophenone and the diphenylbutylpiperidine groups in whole blood, following a Bond Elut extraction. Detection limits in the range 10–100 pg per injection (equivalent to 0.05–0.5 ng/ml whole blood) are reached for the majority of the compounds investigated. With the thioxanthenes the detection limits were found to be

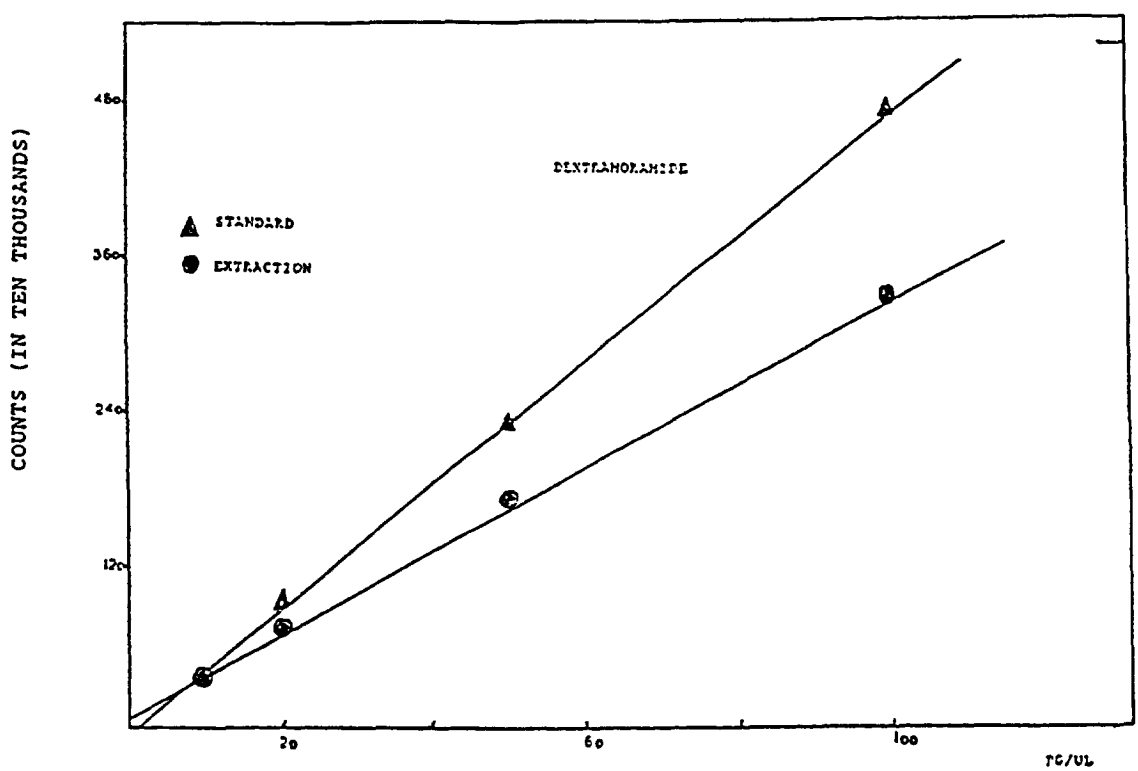

Fig. 1. Methanol calibration graph and data of whole blood spiked with dextromoramide extracted by the Bond Elut method.

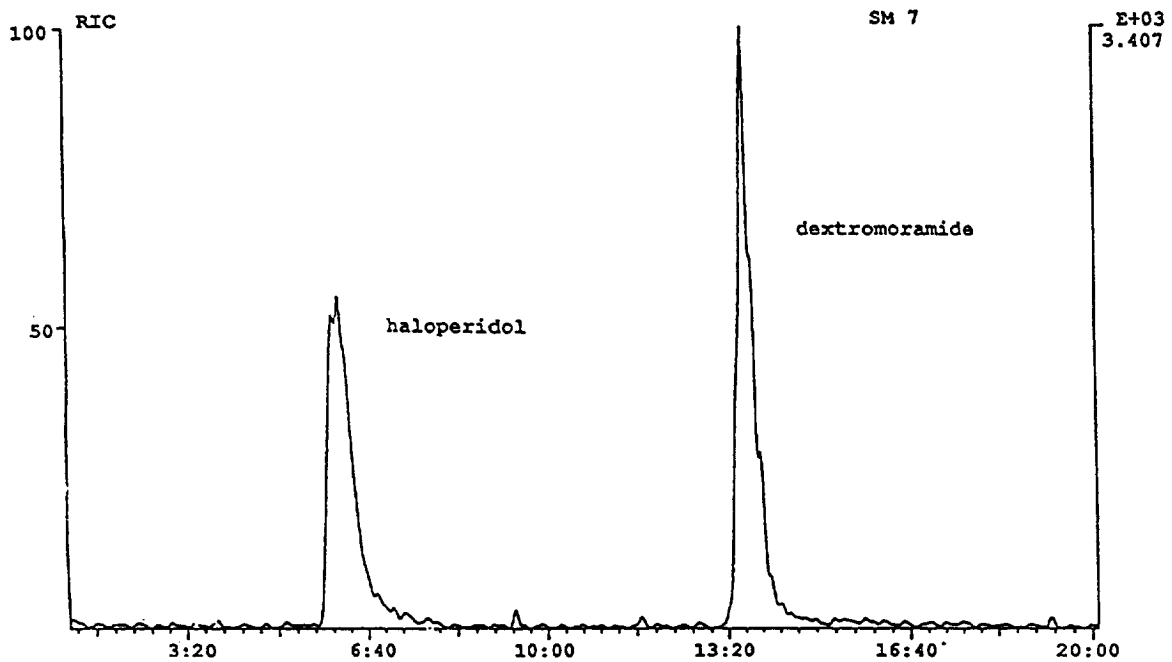

Fig. 2. Chromatogram of 10 μl of an extract of spiked whole blood. A mixture of 500 pg haloperidol and dextromoramide was injected on column.

at least ten times higher, 100–2000 pg per injection (0.5–10 ng/ml whole blood). The method described for whole blood gives a ten to a hundred times better sensitivity than the methods usually applied in the quantitative analysis of these kind of substances.

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