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Immunoaffinity chromatography combined on-line with high-performance liquid chromatography–mass spectrometry for the determination of corticosteroids

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Abstract

On-line coupled immunoaffinity chromatography–reversed-phase high-performance liquid chromatography (IAC–HPLC) with detection by quadrupole ion trap mass spectrometry using a particle beam interface has been developed for the determination of the steroids, dexamethasone and flumethasone. HEMA (polyhydroxyethylmethacrylate) was evaluated as a support material for the anti-dexamethasone antibodies used in IAC. Antibody cross-reactivity and non-specific binding have been investigated for the HEMA bound anti-dexamethasone IAC column. The on-line IAC–HPLC–MS determination of dexamethasone and flumethasone in post-administration equine urine samples showed precisions (R.S.D.) of 8.0 and 7.1%, respectively, with limits of detection in the range 3–4 ng/ml. © 1998 Elsevier Science B.V.

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1. Introduction

The potential of immunoaffinity chromatography–high-performance liquid chromatography–mass spectrometry (IAC–HPLC–MS) has been demonstrated for the determination of analytes at low levels in complex biological matrices [1–3]. The powerful clean-up afforded by the supported antibody in the IAC column, selectively enriching the analyte of interest, compliments the diagnostic power of MS in these procedures. The soft gel supports such as sepharose commonly used in IAC display minimal non-specific binding, but exert certain constraints on their use in a multi-dimensional chromatographic system because they are sensitive to high pressures and flow-rates. The development of more robust antibody support materials would therefore be of great benefit in a multi-dimensional system incorporating IAC. Such a support is HEMA (polyhydroxyethyl methacrylate) a commercially available rigid polymer which has been used in IAC columns for the extraction of LSD [4].

The synthetic corticosteroids are used for their anti-inflammatory effect and are prohibited substances in equine sports. The determination of this class of steroids presents an analytical challenge because their high potency and extensive metabolism results in low concentrations in biological fluids. There are many reports of methods for the determination of the corticosteroids [5–19], which may be classified as screening methods, with limited selectivity, or confirmatory methods involving extensive off-line clean-up prior to analyte determination.

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These compounds are therefore well suited to the development of on-line techniques.

In this paper, we describe the development of an on-line IAC–HPLC–MS method using HEMA as a support for anti-dexamethasone antibodies in an IAC column. Coupling of reversed-phase HPLC with quadrupole ion trap mass spectrometry (QIT-MS) using a particle beam (PB) interface was investigated for the analysis of the corticosteroids, dexamethasone and flumethasone, in post-administration equine urine. The particle beam (PB) is a mechanically simple interface for LC–MS, which allows desolvation and transport of solute molecules to the MS, where they may undergo electron ionisation or chemical ionisation [20]. The nebulisation/desolvation processes of the PB is critical, since the amount of analyte reaching the mass spectrometer ion source is inversely proportional to the heat of vaporisation and heat capacity of the mobile phase [21–23]. Normal-phase HPLC is therefore usually the method of choice for the PB-MS [19], although reversed-phase HPLC has been used successfully with electron impact ionization (EI) as the ionization mode [24]. The sensitivity of the particle beam interface for combined IAC–HPLC under reversed-phase conditions was enhanced in this work by the use of QIT-MS with mass selective ion accumulation of analyte ions during the ionisation step.

2. Experimental

2.1. Materials

Dexamethasone, cortisol, prednisolone deoxymethasone, betamethasone, flumethasone, sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium azide and sodium acetate were purchased from the Aldrich Chemical (Dorset, UK). Propionic acid and methanol (Distol grade) were obtained from Fisons (Loughborough, UK). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All eluents were filtered through 0.45-μm filters (Millipore). HEMA 1000VS-L was purchased from Presearch (Herts, UK). Rabbit serum containing anti-dexamethsone antibody was provided by the Horseracing Forensic Laboratory (Newmarket, UK).

2.2. Standard and sample preparation

Stock solutions of dexamethasone and flumethasone were prepared in methanol at a concentration of 1 mg/ml. Further dilutions were prepared in methanol to give concentrations in the range of 0.2–500 ng/ml for standards analysis and spiking experiments. Spiked and post-administration urine samples (20 ml) were adjusted to pH 7.0 and centrifuged at 1500 *g* for 10 min prior to analysis. The supernatant was removed and 10-ml aliquots were introduced into the IAC–HPLC system.

2.3. Anti-dexamethasone IAC column

The crude rabbit serum samples were purified using a Protein G stationary phase (bed height 4 cm) packed in a C_{10} column (Pharmacia, Uppsala, Sweden). The appropriate fractions were collected and pooled. The antibodies were bound to HEMA and the HEMA was blocked using the method described by the manufacturer [25]. The HEMA was first swollen with water and then washed with 0.1 *M* NaOH/0.5 *M* NaCl, pH 8.3, coupling buffer. The anti-dexamethasone IgG solution (5 ml) was added to the HEMA (1 g) and coupling buffer (5 ml) and the resulting slurry was mixed overnight. The gel was washed and blocked with 0.1 *M* Tris base (pH 9.0). The HEMA anti-dexamethasone stationary phase was packed into a stainless steel HPLC column (50×4.6 mm). The IAC column was stored at 4°C in 30 m*M* phosphate buffer containing 0.5 *M* NaCl+0.2% sodium azide when not in use.

2.4. Instrumentation

2.4.1. IAC–HPLC–UV

The IAC–HPLC–UV instrumentation (Fig. 1a) consisted of a Waters module 6000A HPLC pump (Bedford, MA, USA) (pump 1), which delivered mobile phase to the IAC column via a six-port injection valve (Rheodyne 7010) containing a 20-μl stainless steel injection loop. The mobile phase flowed from the IAC column to V1, a six-port switching valve (Rheodyne 7010) fitted with a 5-ml switching loop (6.4 m×1 mm I.D), which allowed eluted sample factions to be transferred from the IAC column to the HPLC column. Valve V1 was con-

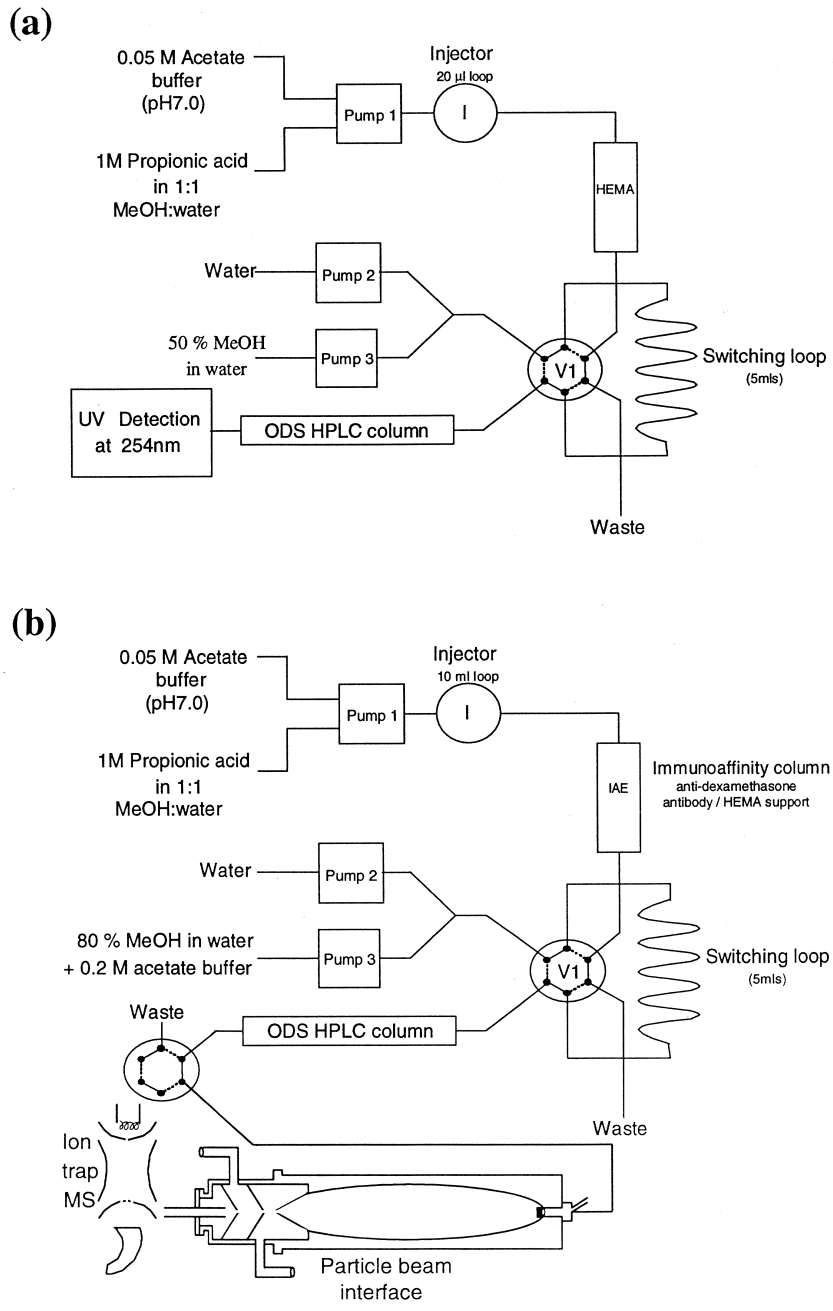

Fig. 1. (a) Schematic diagram of IAC–HPLC–UV system; (b) schematic diagram of IAC–HPLC–PB/QIT-MS system.

nected to two Waters 501 HPLC pumps (pumps 2 and 3). Pump 2 was used to flush the IAC band from the switching loop onto the HPLC column in water, and pump 3 delivered the mobile phase to the HPLC column. The analytical separation was carried out on a 125×4.9 mm I.D. column packed with 5-μm ODS

(Hichrom, Reading, UK), and the effluent was transferred to a Waters Model 441 UV absorbance detector (λ=254 nm).

2.4.2. IAC–HPLC–PB/QIT-MS

The IAC–HPLC–PB/QIT-MS instrumentation (Fig. 1b) used the same IAC–HPLC column switching configuration as that employed for UV detection with the 20-μl injection loop replaced by a 10-ml stainless steel loop (12.7 m×1 mm I.D.). The effluent was transferred from the HPLC column to the particle beam interface. The interface was of modular design [26] and consisted of a heated stainless steel nebuliser, desolvation chamber and two-stage momentum separator, which was differentially pumped to remove solvent. The desolvation chamber was maintained at 50°C with a helium nebulizer pressure of 50 p.s.i. (1 p.s.i.=6894.76 Pa). The interface was coupled to the quadrupole ion trap mass spectrometer (ITMS, Finnigan MAT, San Jose, CA, USA) via a stainless steel transfer line. The QIT-MS system was operated in EI mode, and a filtered noise field (Teledyne Scientific Instruments, Sunnyvale, CA, USA) was used to accumulate analyte ions mass selectively during the ionization period (400 ms).

2.5. Chromatographic procedures

2.5.1. IAC–HPLC–UV

The injected sample (20 μl) containing 0.2–0.8 μg of each of the corticosteroids was delivered to the IAC column in acetate buffer (0.05 *M*, pH 7.0) mobile phase at a flow-rate of 1 ml/min. The IAC column was flushed with mobile phase, with the switching valve (V1) set to the load position, then eluted with methanol–1 *M* propionic acid (50:50, v/v) at 1 ml/min. At 12 min, V1 was switched to the inject position and the recorder was started. The contents of the switching loop were flushed onto the reversed-phase analytical column with water at 3 ml/min. The sample was then eluted with methanol–water (50:50, v/v) at 2 ml/min.

2.5.2. IAC–HPLC–PB/QIT-MS

Mobile phase (0.05 *M* acetate buffer, pH 7.0) was delivered to the IAC column at 2 ml/min and 2×10-ml aliquots of urine were injected. The IAC column was flushed with mobile phase for 15 min and then, with the switching valve (V1) set to the load position, the IAC column was eluted with 50% (v/v) methanol in 1 *M* propionic acid at a flow-rate of 1 ml/min. At 22 min, V1 was switched to the inject position and the contents of the switching loop were flushed onto the reversed-phase analytical column with water at 3 ml/min. The sample was eluted with methanol–0.2 *M* ammonium acetate (80:20, v/v) at 2 ml/min for 3 min with the effluent going to waste. The flow-rate was then reduced to 0.3 ml/min and the eluate switched to the particle beam interface.

3. Results and discussion

3.1. Evaluation of IAC column

HEMA proved to be a robust support material for on-line IAC–HPLC, with the higher back pressures and flow-rates possible through the IAC column in the multidimensional system allowing rapid sample throughput. Soft gel supports such as sepharose, which was used in our earlier studies with anti-dexamethasone antibodies [3], failed under similar flow and pressure conditions. A 5-cm column was used to increase sample protein loading capacity and prolong the lifetime of the HEMA-supported antibodies. The column displayed no deterioration in performance after 200 injections over a 3-month period.

The retention behaviour of the anti-dexamethasone antibodies bound to the HEMA support was investigated for a range of related corticosteroids (Table 1) using UV detection. On loading the anti-dexamethasone antibody-bound HEMA column with the corticosteroids in buffer, followed by elution with methanol–propionic acid, all the steroids were found to be retained to some extent on the IAC column. The length of time taken to flush the column with loading buffer was varied to determine whether this affected the binding of the corticosteroids. The results, given in Table 1, showed that washing the IAC column with buffer reduced significantly the amounts of some of the corticosteroids, notably cortisol and prednisolone, which were detected in the methanol–propionic acid eluting phase. Increased washing with the aqueous loading buffer further

Table 1
Specific and non-specific binding of selected corticosteroids on the anti-dexamethasone antibody/HEMA IAC column

Corticosteroids	Recovery (%) of corticosteroids on the IAC column following flushing with different volumes of washing buffer			
	5 ml	7 ml	10 ml	15 ml
Dexamethasone	80	79	74	73
Cortisol	69	22	nd	nd
Prednisolone	65	24	nd	nd
Deoxymethasone	73	35	20	20
Betamethasone	71	37	22	21
Flumethasone	81	76	71	72

0.2–0.8 μg of each corticosteroid loaded onto the IAC column. nd, none detected.

reduced the amounts of these two corticosteroids until, after 10 min washing of the column, no residual analyte was detected in the eluting band of the IAC column. Under similar conditions the recoveries for deoxymethasone and betamethasone were 20–22%, and those for dexamethasone and flumethasone were >70%. This suggested that there was considerable non-specific binding on the IAC column, in addition to specific antibody–antigen binding, but these non-specific interactions between the column and the corticosteroids were sufficiently weak that adsorbed compounds could be removed quantitatively by washing with aqueous buffer.

The contribution of the support material to the non-specific binding was investigated for the adsorption of the corticosteroids on HEMA using the same sample loading, washing and elution procedures. The system shown in Fig. 1a was used with the anti-dexamethasone antibody–HEMA IAC column being replaced with a HEMA column. The results, given in Table 2, confirmed that non-specific binding by HEMA was occurring for all the corticosteroids, but that washing with 10 ml of loading buffer was sufficient to remove the support-bound analyte.

Table 2
Non-specific binding of selected corticosteroids on HEMA support

Corticosteroids	Recovery (%) of corticosteroids on the HEMA column following flushing with different volumes of washing buffer			
	5 ml	7 ml	10 ml	15 ml
Dexamethasone	62	30	nd	nd
Cortisol	54	28	nd	nd
Prednisolone	69	36	nd	nd
Deoxymethasone	57	25	nd	nd
Betamethasone	64	56	nd	nd
Flumethasone	61	28	nd	nd

nd, none detected.

If the amount of each of the corticosteroids retained on the IAC column containing anti-dexamethasone antibodies, after washing with 10 ml or more of aqueous buffer, is assumed to be due to specific binding by the anti-dexamethasone antibodies and not due to non-specific adsorption, then cross-reactivity for the corticosteroids may be determined. The calculated cross-reactivities, for the IAC column relative to dexamethasone, are similar to those determined by enzyme-linked immunosorbent assay (ELISA) (Table 3) [27]. The observed cross-reactivities suggest that the anti-dexamethasone IAC column may be suitable for the quantitative determination of dexamethasone and flumethasone, and may also be useful to screen for deoxymethasone and betamethasone, but that there was no significant binding of cortisol and prednisolone.

3.2. IAC–HPLC–PB/QIT-MS

The IAC–HPLC pre-treatment, using a switching loop to transfer the steroid-containing fraction eluted from the IAC column to the HPLC column, was coupled via a particle beam interface to the quadrupole ion trap mass spectrometer (PB/QIT-MS). The EI mass spectrum of dexamethasone eluted from the HPLC column produced a prominent fragment ion at m/z 312, whilst flumethasone gave a characteristic ion at m/z 350, and these ions were selected for monitoring the two corticosteroids by IAC–

Table 3
Cross-reactivities for selected corticosteroids using IAC–HPLC

Corticosteroid	Relative cross-reactivity (%)	
	IAC–HPLC	ELISA
Dexamethasone	100	100
Flumethasone	96	96
Betamethasone	30	37
Deoxymethasone	27	21
Cortisol	0	1
Prednisolone	0	4

Antiserum batch: AD60.

HPLC–PB/QIT-MS. The major drawback of using the particle beam was that a high proportion of the analyte was lost during passage through the interface and this meant that the conditions for the particle beam needed to be carefully tuned to achieve the levels of detection required for biological assay. To detect the corticosteroid dexamethasone by QIT-MS, a filtered noise field with a notch at 32.75–34.50 kHz was applied to the end caps to eject solvent and matrix ions from the ion trap during ionization. A long ionization time was used to allow a population of m/z 312 ions to accumulate in the trap, so increasing the sensitivity of the detector. Optimum transport of the corticosteroid through the PB interface was found to be achieved with a high percentage of methanol and the addition of a carrier, ammonium acetate, to the mobile phase. With a concentration of 0.2 *M* ammonium acetate buffer in the 80% methanol–water mobile phase, 100 ng of dexamethasone could be detected by PB/QIT-MS with a >3:1 signal-to-noise ratio.

Dexamethasone standards spiked in equine urine in the range of 100–500 ng were analysed by IAC–HPLC–PB/QIT-MS. A linear response curve was obtained, with a correlation coefficient of 0.993. Selected ion monitoring (SIM) of the m/z 312 ion and the chromatographic retention time were used to confirm the presence of dexamethasone. The IAC–HPLC–PB/QIT-MS system had a limit of detection calculated at 3 ng/ml (signal:noise=2:1) and sequential replicate injections of a spiked urine sample (10 ng/ml) showed a relative standard derivation of 7.4%. The relative standard derivation for a 10-ng/ml sample injected over a period of 7 days was 8.0% (n=5). Fig. 2a shows the chromatogram from a sample collected 1.5 h after a single intra-muscular injection (20 μg/kg) of a Dextran preparation of dexamethasone to a horse. The concentration of dexamethasone in this sample was determined as 9 ng/ml.

Flumethasone was determined using a notch set at 30.50–28.50 kHz to trap the m/z 350 ions as the analyte eluted from the HPLC column. The instrumental conditions were otherwise the same as those for the dexamethasone determination. A series of flumethasone-spiked standards were run in the range 100–500 ng, giving a linear response curve (correlation coefficient 0.989). SIM of the m/z 350 ion and the chromatographic retention time were

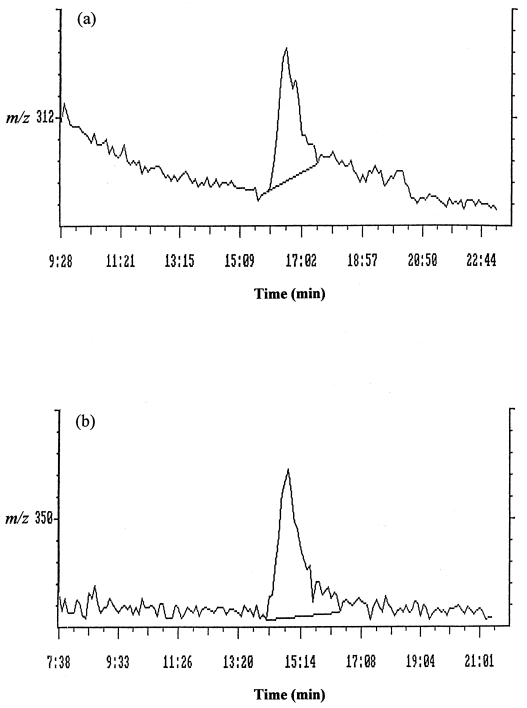

Fig. 2. On-line IAC–HPLC–PB/QIT-MS determination of (a) dexamethasone in a sample collected 1.5 h after a single intramuscular injection of a Dextran preparation and (b) flumethasone in a sample collected 1.85 h after a single intra-muscular injection of a Flucort preparation (see Section 2 for chromatographic conditions).

used to confirm the presence of flumethasone. The IAC–HPLC–PB/QIT-MS system had a limit of detection calculated at 4 ng/ml (signal:noise=2:1). Replicate injections of a spiked sample using (15 ng/ml) showed a relative standard derivation of 6.9%. This increased slightly to 7.1% (n=5) when calculated for flumethasone samples injected over 7 days. Fig. 2b shows the chromatogram from a sample collected 1.85 h after a single intra-muscular injection (4.8 μg/kg) of a Flucort preparation of flumethasone to a thoroughbred horse. The concentration of flumethasone in this sample was determined as 10 ng/ml.

EI produced significant fragmentation of the corticosteroids under investigation and this, combined with the poor transmission of analyte through the particle beam interface, resulted in limits of detection of 3–4 ng/ml using selected ion monitoring, although full scan data required for confirmatory analysis may be acquired with little loss in sensitivity

because of the ion storage capabilities of the ion trap. The detection limits that would be required for analysis of corticosteroids at lower levels could be achieved by increased sample size, since the high selectivity of IAC allows large sample volumes to be handled without loss of analytical performance. The on-line combination of IAC–HPLC–MS eliminated the need for the lengthy and complex extraction and derivatisation procedures which are necessary when analytes are determined using IAC followed by off-line derivatisation and GC–MS. The methodology is also simpler in terms of sample manipulation and, with less manual intervention, there are fewer stages where analyte losses or contamination may occur. The rigid HEMA polymer support allowed faster sample loading and elution of the IAC column, reducing analysis times compared to the soft gel-supported IAC approach [3].

4. Conclusion

On-line IAC–HPLC–PB/QIT-MS has been applied to the analysis of early post administration equine urine samples with high precision and without prior sample pre-treatment. The HEMA support material proved more robust and convenient to use than soft gel supports for IAC. Automation of the method would be straightforward for the routine confirmatory analysis of corticosteroids in the equine.

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References

[1] G.S. Rule, J.D. Henion, J. Chromatogr. 582 (1992) 102.
[2] E. Davoli, R. Fanelli, R. Bagnati, Anal. Chem. 65 (1993) 2679.
[3] C.S. Creaser, S.J. Feely, E. Houghton, M. Seymour, P. Teale, Anal. Commun. 33 (1996) 5.
[4] D.J. Hawkes, in: 17th Int. Symposium on Capillary Chromatography and Electrophoresis, Wintergreen, VA, 1995, p. 137.
[5] D.I. Chapman, M.S. Moss, J. Whiteside, J. Vet. Rec. 100 (1977) 447.
[6] E. Houghton, M.C. Dumasia, J.K. Wellby, Biomed. Mass Spectrom. 8 (1981) 558.
[7] E. Houghton, P. Teale, M.C. Dumasia, Analyst 109 (1984) 273.
[8] S.J. Gaskell, L. Sieckman, Clin. Chem. 32 (1996) 536.
[9] M. Ishibashi, H. Takayama, Y. Nakagawa, N. Harima, Chem. Pharm. Bull. 36 (1988) 845.
[10] P.J. O'Connor, W.P. Morgan, S.J. Raglin, C.M. Barksdale, N.H. Kalin, Psychoneuroendocrinology 14 (1989) 303.
[11] C.J. Maclean, C.W. Booth, T. Tattersall, J.D. Few, Eur. J. Appl. Physiol. 58 (1989) 341.
[12] J. Park, S. Park, D. Lho, H.P. Choo, B. Chung, C. Yoon, H. Min, M.J. Choi, Anal. Toxicol. 4 (1990) 66.
[13] J. Shibaski, T. Furata, Y. Kasuya, T. Okase, T. Katoh, T. Kogo, T. Hirayama, Biomed. Environ. Mass Spectrom. 19 (1990) 225.
[14] B.K. Jap, G.A.R. Johnston, R. Kazlauskas, J. Chromatogr. 573 (1992) 183.
[15] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. 579 (1992) 193.
[16] S.M.R. Stanley, B.S. Wilhelmi, J.P. Rodgers, J. Bertschinger, J. Chromatogr. 614 (1993) 77.
[17] S.M.R. Stanley, B.S. Wilhelmi, J.P. Rodgers, J. Chromatogr. 620 (1993) 250.
[18] D. Courtheyn, J. Vercammen, H. De Brabander, I. Vandenreyt, P. Batjoens, K. Vanoosthuyze, C. Van Peteghem, Analyst 119 (1994) 2557.
[19] S.M.R. Stanley, B.S. Wilhelmi, J.P. Rodgers, A. Guthrie, Biol. Mass Spectom. 23 (1994) 483.
[20] C.S. Creaser, J.W. Stygall, Analyst 118 (1993) 1467.
[21] A.P. Tinke, R.A.M. van der Hoeve, W.M.A. Niessen, U.R. Tjaden, J. van der Greef, J. Chromatogr. 554 (1991) 119.
[22] V. Raverdino, J. Chromatogr. 554 (1991) 125.
[23] R.D. Voyskner, C.S. Smith, P.C. Knox, Biol. Mass Spectrom. 19 (1990) 523.
[24] C. Julian-Larose, P. Voirin, C. Mas-Chamberlin, A. Dufour, J. Chromatogr. 614 (1993) 39.
[25] HEMA support, Presearch, 1993.
[26] C.S. Creaser, J.W. Stygall, Inst. Sci. Technol. 22 (1994) 185.
[27] C.J. Roberts, L.S. Jackson, J. Immunol. Methods 181 (1995) 157.