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Journal of Chromatography B, 738 (2000) 17–25

JOURNAL OF CHROMATOGRAPHY B

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Simultaneous determination of hydrocortisone, dexamethasone, indomethacin, phenylbutazone and oxyphenbutazone in equine serum by high-performance liquid chromatography

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Received 29 June 1999; received in revised form 13 October 1999; accepted 13 October 1999

Abstract

Ethyl acetate extracts of equine serum, containing 0–5 μg/ml of hydrocortisone (HYD), dexamethasone (DEX), oxyphenbutazone (OPB), indomethacin (IND), phenylbutazone (PB) and probenecid as internal standard, were evaporated with nitrogen, resuspended in methanol and analyzed by HPLC, using a C-18 column equilibrated with 51:49 acetonitrile–water, 0.1% trifluoroacetic acid, at 1 ml/min. The eluate was monitored at 254 nm. The selectivity (inter-assay C.V.<4%), sensitivity (limits of quantitation of 0.25 μg/ml for HYD, DEX and IND, 0.5 μg/ml for PB and 1 μg/ml for OPB, despite the occurrence of significant degradation of OPB and PB during the analysis) and precision (intra-assay and inter-assay C.V.'s of about 3–6 and 9–15%, respectively) of the method appeared appropriate for anti-doping control of racehorses.

Keywords: Hydrocortisone; Dexamethasone; Indomethacin; Phenylbutazone; Oxyphenbutazone

1. Introduction

Steroidal and non-steroidal anti-inflammatory drugs (NSAID), such as hydrocortisone (HYD), dexamethasone (DEX), and phenylbutazone (PB), are commonly used in numerous musculo-skeletal inflammatory conditions of the horse [1–3]. However, the competition of treated racehorses is prohibited by several regulations in order to prevent health problems which can lead to the premature withdrawal of the animal from athletic life [4,5]. Actually, the use of PB is allowed by some racing jurisdictions but serum levels should not exceed 2–8 μg/ml [5–8].

To the best of our knowledge, there is no HPLC method capable of determining simultaneously HYD, DEX, oxyphenbutazone (OPB), indomethacin (IND) and PB in equine serum. Therefore, we decided to develop a method for the analysis of these anti-inflammatory drugs during anti-doping controls.

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0378-4347/00/$ – see front matter
PII: S0378-4347(99)00478-8

2. Experimental

2.1. Chemicals and reagents

Hydrocortisone (HYD, 98% pure), dexamethasone (DEX, 98% pure), oxyphenbutazone (OPB, 99% pure), probenecid (PRB, 99.8% pure), indomethacin (IND, 99% pure), phenylbutazone (PB, 99.7% pure), sodium citrate (99% pure), sodium phosphate (99% pure) and normal equine serum (NES) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile and water, were purchased from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid (99.8% pure) was from Merck (Darmstadt, Germany). Ethyl acetate (99.5% pure) was obtained from Carlo Erba (Milan, Italy).

2.2. Standard solutions

Standard solutions of HYD, DEX, OPB, PRB, IND and PB, prepared by dissolving separately the drugs in methanol at the concentration of 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml, were stored at −20°C until used, with the exception of the OPB and PB solutions, which were always prepared separately on the same day of analysis.

2.3. Administration procedure

Three adult horses were administered an intravenous bolus of PB (Bute®), at the dose of 4.4 mg/kg, and jugular venous blood samples were withdrawn at 5 min, 5 h and 24 h after the injection, immediately centrifuged at 2000 *g* for 5 min and stored at −20°C until used.

2.4. Serum samples processing

To 1 ml of NES, spiked (0–5 μg/ml) with HYD, DEX, OPB, IND and PB, or serum obtained from horses treated or not with PB, 50 μg (10 μl of a methanolic solution 5 mg/ml) of the internal standard PRB were added before each analysis. Afterwards, samples were extracted with 2 ml of ethyl acetate and an aliquot of 1 ml of the organic phase was withdrawn, evaporated to dryness under a nitrogen stream at room temperature and redissolved in 300 μl of methanol. Hence, 50 μl of these solutions were analyzed by HPLC.

2.5. High performance liquid chromatography

The chromatographic system consisted of a controller (Pharmacia-LKB, model LCC 2252), a precision pump (Pharmacia-LKB, model 2248) a high pressure mixer, a Rheodyne loop injector, a programmable photodiode array detector (Waters, model 994) or a UV-monitor (Pharmacia-LKB, model 2151), and a peak area integrator (Pharmacia-LKB, model 2221).

An isocratic method capable of separating HYD, DEX, OPB, PRB, IND, and PB by reversed-phase HPLC was developed, using a C-18 column (4.6× 250 mm; 5 μm particle size; 80 Å pore size, Beckman cat. no. 235329). The column was equilibrated at the flow-rate of 1 ml/min with a mobile phase consisting of acetonitrile in water (51:49, v/v), containing 0.1% trifluoroacetic acid. The eluate was monitored at 254 nm. All chromatographic analyses were carried out at room temperature. The average column back pressure was about 7 MPa.

2.6. Validation procedures

2.6.1. Selectivity

The selectivity of the method was determined based on multiple analyses of a mixture of HYD, DEX, OPB, PRB, IND and PB, separately suspended in methanol or NES. All drugs but PRB (50 μg/ml) were dissolved at the concentration of 5 μg/ml.

Besides, the effect of pH on the selectivity of the method was evaluated by analyzing NES spiked with HYD, DEX, OPB, IND, PB (5 μg/ml) and PRB (50 μg/ml) before and after adjustment of the pH at 4 using 0.345 *M* sodium citrate, pH 2.3.

2.6.2. Sensitivity

A 10 μl volume of the standard solutions of HYD, DEX, OPB, PRB, IND and PB were added to 1 ml of NES and the samples were processed and analyzed by HPLC as described above:

- the limit of detection corresponded to a signal-to-noise ratio of 2:1;
- for each drug, the area under the peak was automatically integrated, plotted versus the corresponding concentration and a linear regression analysis was performed using the software Sigma-

Plot 5.0 (SPSS, Chicago, IL, USA). The limit of quantitation corresponded to the lowest concentration that could be determined with a coefficient of variation (C.V.) less than 15%.

2.6.3. Precision

A quality control (QC) sample was prepared by adding 1 ml of a methanolic solution of HYD, DEX, OPB, IND and PB (500 μg/ml, each dissolved separately) to 99 ml of NES. Hence, this sample was divided in aliquots of 1 ml which were stored at −20°C until used. For each assay, a different aliquot was employed and analyzed in duplicate. The concentration of each drug was determined and the intra- and inter-assay coefficients of variations (C.V.) were calculated.

2.6.4. Recovery

The absolute recovery of the extraction of HYD, DEX, OPB, IND, PB (0–5 μg/ml) and PRB (0–50 μg/ml), from equine serum was determined by dividing the slope of the standard curves obtained by analyzing NES spiked with the drugs by the slope of the corresponding methanolic solutions, according to the formula:

$$\text{Absolute recovery (\%)} = \frac{(\text{slope})_{\text{plasma}}}{(\text{slope})_{\text{methanol}}} \cdot 100$$

3.1. Oxyphenbutazone and phenylbutazone degradation

3.1.1. Effect of degradation on the UV spectrum of phenylbutazone

A 50 μl volume of a methanolic solution of PB (10 μg/ml) were added to 950 μl of:

(a) methanol;
(b) ethyl acetate, and the sample was evaporated under a nitrogen stream at room temperature and resuspended in 1 ml of methanol;
(c) ethyl acetate, and the sample was evaporated as in (b), kept at 100°C for 30 min, cooled in an ice bath for 30 s, and resuspended in 1 ml of methanol;

and the UV spectra were obtained using a UV/Vis spectrophotometer (model Lambda Bio 20, from Perkin-Elmer Co., Norwalk, USA).

3.1.2. Influence of degradation on the overall recovery of phenylbutazone

A 10 μl volume of methanolic solutions (0, 0.1, 0.2 and 0.4 μg/μl) of PB were:

(a) added to 590 μl of methanol;
(b) added to 2 ml of ethyl acetate and an aliquot of 1 ml was withdrawn and evaporated under a nitrogen stream at room temperature and resuspended in 300 μl of methanol;
(c) added to 1 ml aliquots of NES, and the samples were incubated for 20 min at room temperature, extracted with 2 ml of ethyl acetate and 1 ml of the organic phase was withdrawn, evaporated as in (b) and resuspended in 300 μl of methanol;
(d) added to 1 ml aliquots of PBS (10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4 at 22°C) and the samples were processed as in (c);

and of 50 μl of these methanolic solutions were analyzed by HPLC.

3.1.3. Effect of pH on the recovery of oxyphenbutazone and phenylbutazone

OPB and PB were dissolved in either PBS, pH 7.4 or 0.345 *M* sodium citrate, pH 2.3 at different concentrations in the range 0–100 μg/ml and the absorbance at 254 nm of these solutions was measured before (A_{before}) and after (A_{after}) the extraction with ethyl acetate, using the UV/Vis spectrophotometer described above. Hence, the yields (Y) of extraction were calculated with the formula $Y = (1 - A_{\text{after}}/A_{\text{before}}) \cdot 100$.

3.1.4. Phenylbutazone degradation in the autosampler

PB was suspended in 12 different aliquots of 1 ml of NES at the concentration of 5 μg/ml and prepared for the HPLC analysis as described above. Then, the methanolic solutions were analyzed either immediately or incubated for 30 h at 22°C or 72 h at 4°C prior to the injection into the chromatograph.

4. Results

4.1. High-performance liquid chromatography

4.1.1. Selectivity

When 50 μl of methanolic solutions (5 μg/ml)

containing hydrocortisone (HYD), dexamethasone (DEX), oxyphenbutazone (OPB), probenecid (PRB), indomethacin (IND) and phenylbutazone (PB) were analyzed by reversed-phase HPLC, using a C-18 column with a mobile phase containing about 50% acetonitrile in water, the chromatogram shown in Fig. 1 was obtained. It was noted that the peaks were well separated even following extraction of the drugs from equine serum without significant interference of co-extracted components from the matrix (Figs. 2A and 3, Table 1).

When commercially available normal equine serum (NES), spiked with HYD, DEX, OPB, IND, PB (5 μg/ml) and PRB (50 μg/ml) was extracted with ethyl acetate before and after adjustment of pH at 3.4, and analyzed by HPLC, two different chromatograms were obtained (Fig. 2A and B). It was noted that the areas under the peaks of HYD, DEX, OPB and especially PRB, but not that of IND and PB, were significantly increased following acidification of the sample (Fig. 2B vs. 2A, 2C). However, at low (Fig. 2B) but not at neutral (Fig. 2A) pH, co-extracted matrix components interfered with the detection of HYD (Fig. 2A and B, peak 1).

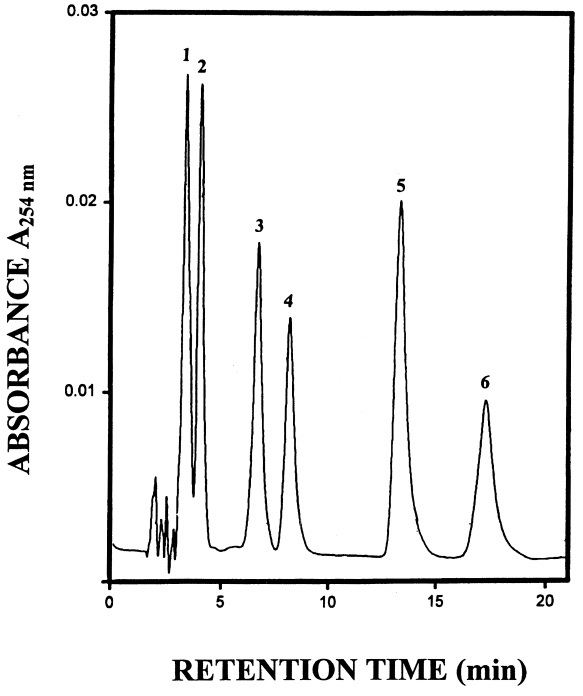

Fig. 1. Analysis of methanolic standards. (A) 50 μl of methanolic solutions of hydrocortisone (5 μg/ml, peak 1), dexamethasone (5 μg/ml, peak 2), oxyphenbutazone (5 μg/ml, peak 3), probenecid (50 μg/ml, peak 4), indomethacin (5 μg/ml, peak 5) and phenylbutazone (5 μg/ml, peak 6) were loaded onto a C-18 column equilibrated (1 ml/min) with acetonitrile–water 51:49 (v/v), 0.1% TFA, and the eluate was monitored at 254 nm.

4.1.2. Sensitivity

The analysis of NES spiked with different concentrations (0–5 μg/ml) of HYD, DEX, OPB, IND and PB, yielded the chromatograms shown in Fig. 3. The limits of detection are reported in Table 1.

When we divided the areas under the peaks shown in Fig. 3 by the peak area of the internal standard PRB (50 μg/ml, peak 4), calibration curves with correlation coefficients of 0.99 were obtained, indicating a good linear regression. The limits of quantitation are reported in Table 1.

4.1.3. Precision

The intra- and inter-assay coefficients of variations (C.V.) are reported in Table 1.

4.1.4. Recovery

The absolute recoveries of the extraction from NES, using ethyl acetate, were high for HYD, DEX, and IND, low for OPB and PB and very low for PRB (Table 1).

4.2. Serum levels of phenylbutazone and oxyphenbutazone following intravenous administration in the horse

When three adult horses were treated intravenously with PB (4.4 mg/kg), the drug was detected in their serum for more than 24 h (Fig. 4A, peak 6). The serum concentration versus time plots for PB and its major metabolite, OPB (Fig. 4A, peak 3), are depicted in Fig. 4B and C, respectively.

4.3. Oxyphenbutazone and phenylbutazone degradation

4.3.1. Influence of degradation on the UV spectrum of phenylbutazone

The methanolic spectra of undegraded (I), partially degraded (II, evaporated from an ethyl acetate solution) or extensively degraded (III, corresponding to II incubated for 30 min at 100°C) PB, are shown in Fig. 5A. Significant differences were noted between the spectra of I and II (in the range 200–254 nm) and I and III (in the range 200–300 nm).

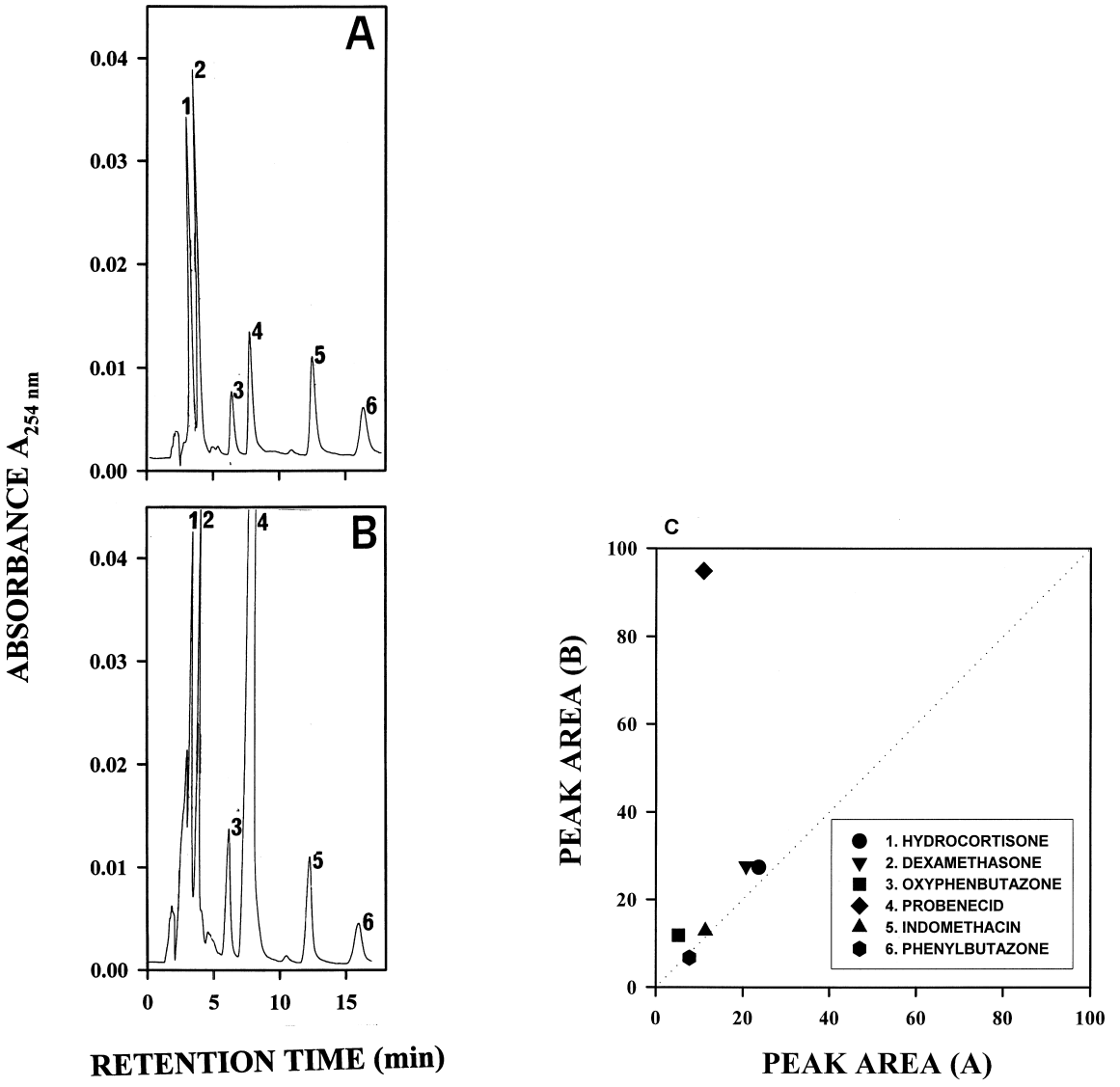

Fig. 2. Selectivity of the method at neutral and low pH. Commercially available normal equine serum (NES) was spiked with hydrocortisone (5 μg/ml, peak 1), dexamethasone (5 μg/ml, peak 2), oxyphenbutazone (5 μg/ml, peak 3), probenecid (50 μg/ml, peak 4), indomethacin (5 μg/ml, peak 5) and phenylbutazone (5 μg/ml, peak 6) and the samples were analyzed by HPLC before (A) and after (B) adjustment of pH at 4 using 0.345 *M* sodium citrate, pH 2.3. (C) The areas of the peaks shown in (A) were plotted against those of (B).

4.3.2. Influence of degradation on the overall recovery of phenylbutazone

The slope of the standard curve obtained by analyzing the extracts of NES spiked with 0, 1, 2 and 4 μg/ml of PB (Fig. 5B, plot III) was very similar to that obtained using PBS (Fig. 5B, plot IV) but much lower than that resulting from the analysis of equivalent amounts of the drug directly dissolved in methanol (Fig. 5B, plot I). Besides, it was noted that the dissolution of the drug in ethyl acetate and the subsequent evaporation process caused a significant decrease of the slope (Fig. 5B, plot II vs. plot I).

4.3.3. Effect of pH on the recovery of oxyphenbutazone and phenylbutazone

When the overall recoveries of PB and OPB

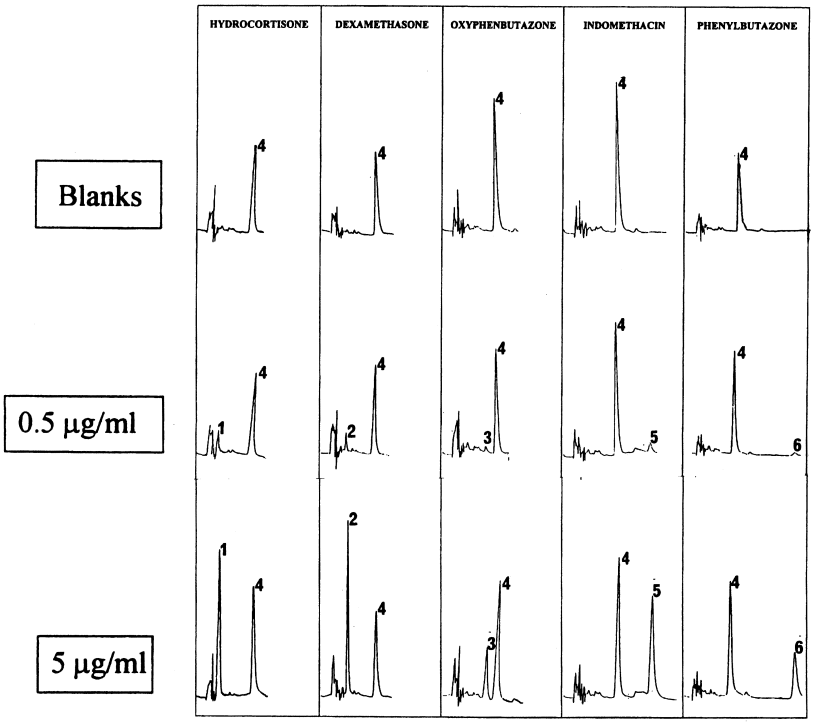

Fig. 3. Analysis of equine serum standards. Analysis of aliquots of 1 ml of commercially available normal equine serum (NES) spiked with different drugs at 0.5 and 5 μg/ml.

(Table 1, Fig. 5C, plot I), which are relative to the complete process of solvent extraction and evaporation, were compared to the recoveries of the extraction from PBS, pH 7.4 and 0.345 *M* sodium citrate, pH 2.3 (Fig. 5C, plot II and III, respectively, obtained with a different method which did not require the evaporation of the solvent, significant differences were observed.

4.3.4. Phenylbutazone degradation in the autosampler

Following the extraction of PB from NES (5

Table 1
Analysis of selected steroidal and non-steroidal anti-inflammatory drugs by HPLC

Drug	Selectivity			Sensivitity (μg/ml)		Precision		Recovery
	R_t[a] (min)	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Detection limit[b]	Quantitation limit[c]	Intra-assay C.V. (%)	Inter-assay C.V. (%)	(%)
Hydrocortisone	3.5	0.9 ($n=2$)	3.3 ($n=30$)	0.25	0.25	6.5 ($n=2$)	9.5 ($n=30$)	89.0±7.8
Dexamethasone	4.0	0.9 ($n=2$)	1.7 ($n=30$)	0.25	0.25	5.7 ($n=2$)	9.9 ($n=30$)	88.2±0.4
Oxyphenbutazone	6.8	1.1 ($n=2$)	2.0 ($n=30$)	0.5	1	2.7 ($n=2$)	13.6 ($n=30$)	37.9±0.9
Probenecid	8.3	0.9 ($n=2$)	1.8 ($n=30$)	–	–	4.6 ($n=2$)	8.7 ($n=30$)	6.5±0.9
Indomethacin	13.4	0.5 ($n=2$)	1.7 ($n=30$)	0.25	0.25	6.1 ($n=2$)	13.9 ($n=30$)	78.1±5.5
Phenylbutazone	17.4	0.5 ($n=2$)	4.1 ($n=30$)	0.5	0.5	5.5 ($n=2$)	14.8 ($n=30$)	51.5±2.7

[a] Retention time.
[b] Corresponding to a signal-to-noise ratio of 2:1.
[c] Defined as the concentration yielding a peak whose area could be quantitated with a C.V. lower than 15%.

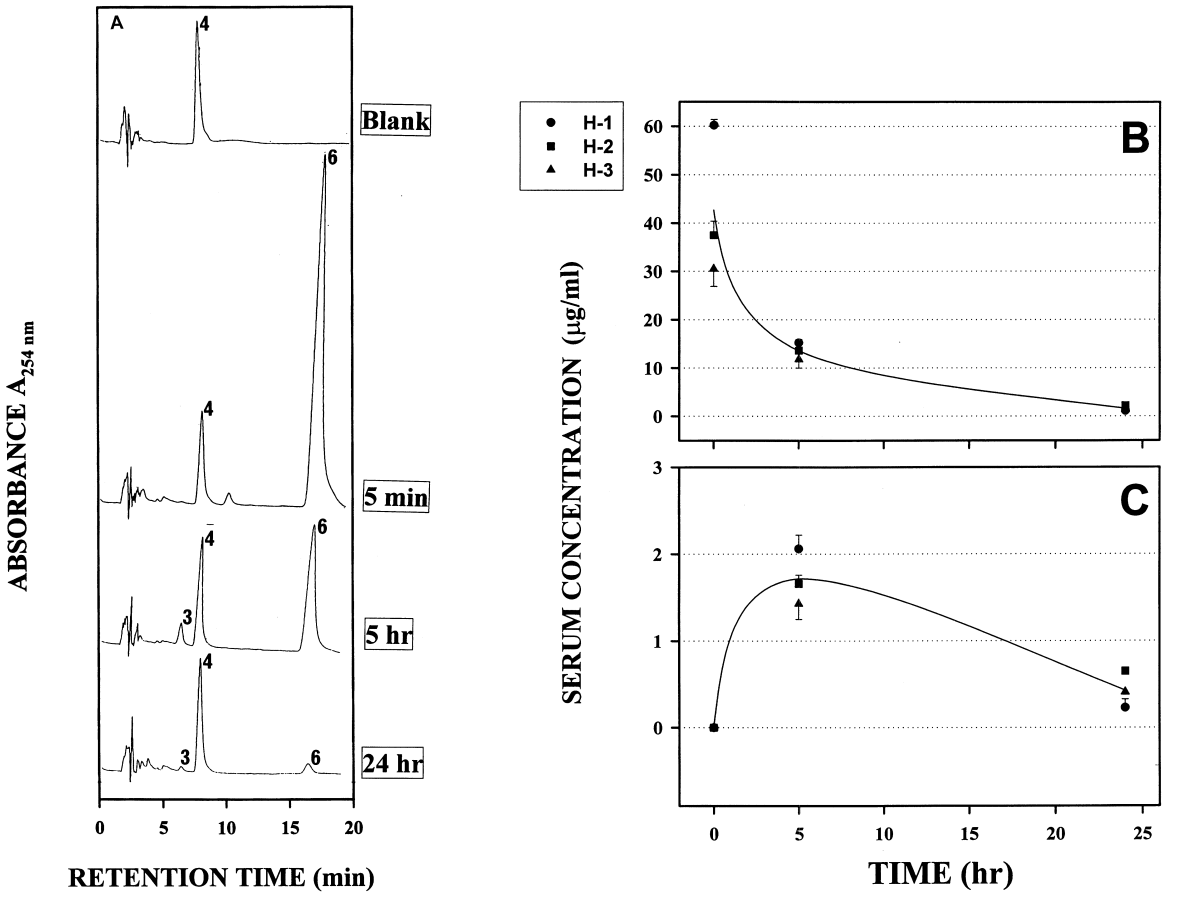

Fig. 4. Analysis of serum samples obtained from horses treated with phenylbutazone. (A) Analysis of serum obtained from horses treated intravenously with PB at the dose of 4.4 mg/kg: peak 3: oxyphenbutazone; peak 4: probenecid; peak 6: phenylbutazone. Endogenous hydrocortisone was not detectable. (B, C) Time course of phenylbutazone (B) and oxyphenbutazone (C) for three different animals (H-1, H-2 and H-3).

μg/ml), the incubation for 30 h at 22°C or 72 h at 4°C of the methanolic samples before the HPLC analysis, did not significantly (according to the ANOVA test) affect the results (Fig. 5D).

5. Discussion

A quantitative, reversed-phase HPLC method, for the simultaneous analysis of selected steroidal and non-steroidal anti-inflammatory drugs in equine serum, with limits of quantitation [0.25 μg/ml for HYD, DEX and IND, 0.5 μg/ml for PB and 1 μg/ml for OPB (Table 1)] suitable for anti-doping control, was described.

Ethyl acetate, a non-toxic solvent, was preferred over others, such as acetonitrile, ethanol, trichloroacetic and perchloric acid because of the better yield and selectivity (data not shown). We did not find very time consuming the liquid–liquid extraction step which does not involve any centrifugation and, if necessary, can be accomplished using a multiple-tube evaporator such as the Evap-O-Rac System (Cat. no. 01610-15) from Cole Parmer (Vernon Hills, IL, USA). Serum samples were not acidified prior to the extraction, as described in many methods for anti-inflammatory drugs, because the recoveries of HYD, DEX and IND (Table 1), were judged satisfactory and, unexpectedly, no improvement was noted for PB (Fig. 2B and C). To explain the latter result,

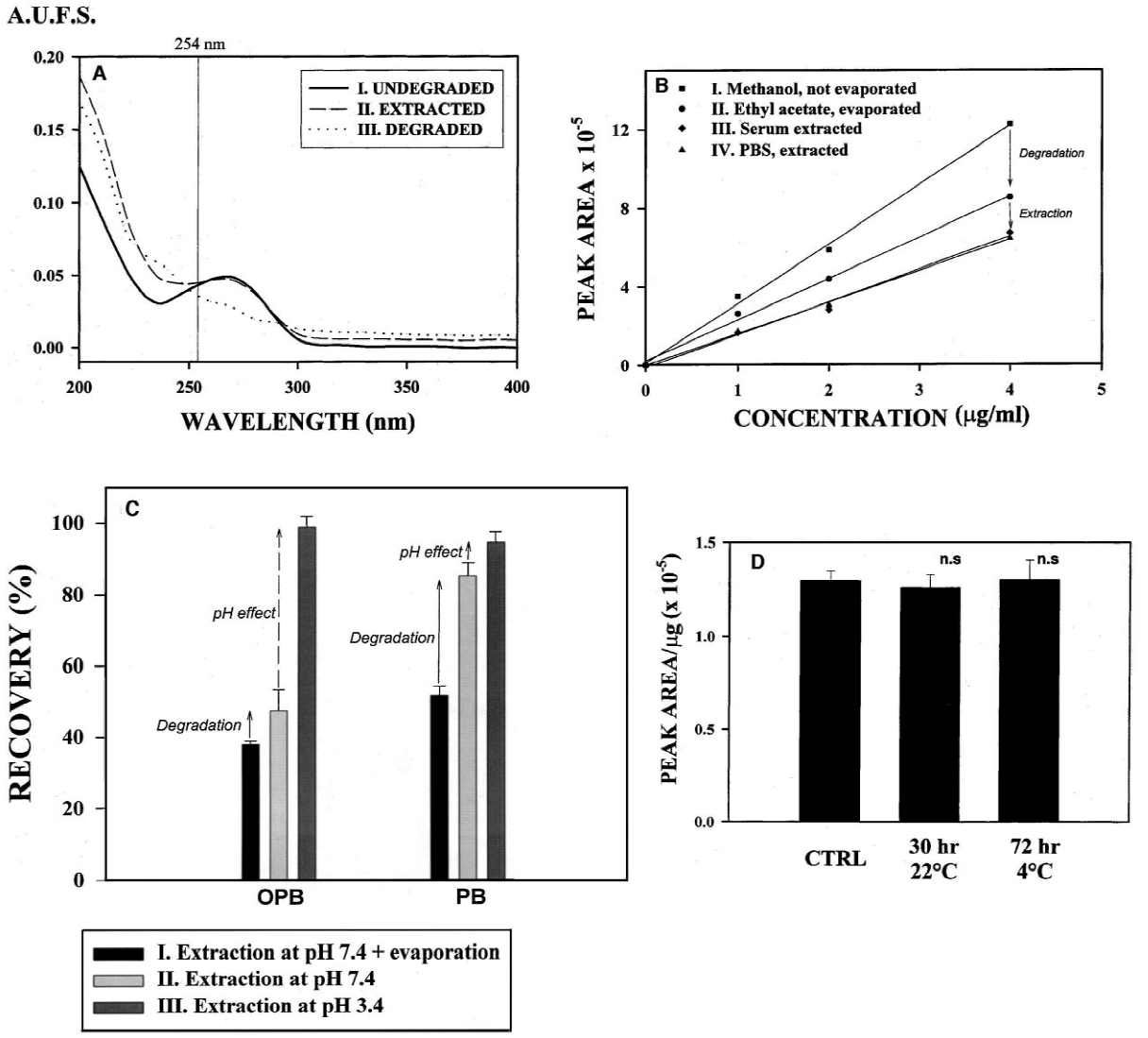

Fig. 5. Degradation of phenylbutazone and oxyphenbutazone. (A) UV spectra of 500 μg of phenylbutazone (PB) dissolved in: 1 ml of methanol (I); ethyl acetate, evaporated and resuspended in 1 ml of methanol (II); ethyl acetate, evaporated, kept at 100°C for 30 min and resuspended in 1 ml of methanol (III). (B) Analysis PB directly dissolved in methanol (I); dissolved in ethyl acetate, evaporated and resuspended in methanol (II); suspended in commercially available equine serum (NES), extracted with ethyl acetate (III), and processed as II (III); dissolved in phosphate buffered saline (PBS) and processed as III (IV). (C) The recovery of the extraction of PB was estimated by HPLC (I) or by conventional spectrophotometry (II) skipping the evaporation step as detailed in the text. (D) Aliquots (1 ml) of NES spiked with PB (5 μg/ml) were processed for the analysis and the resulting methanolic solutions were injected into the HPLC either immediately or after incubation for 30 h at 22°C or 72 h at 4°C. Data ($n=4$) are mean±SD n.s., non-significant by the ANOVA test, performed with the software Sigma-Stat version 2.0 for Windows 95 (SPSS, Chicago, IL, USA).

we speculated that the increase of the recovery of PB following acidification (shown in Fig. 5C) was counterbalanced by an accelerated degradation of the drug at low pH [9,10], which appeared significant (Fig. 5C), despite the use of citric acid instead of hydrochloric acid as recommended by Caturla and Cusido [11]. Besides, from acidified serum, additional components were extracted, which interfered with the detection of HYD (Fig. 2B). Incidentally, we noted that serum acidification was associated with a

quantitative variation of the internal standard PRB (Fig. 2B vs. 2A, peak 4), due to the dramatic increase of the recovery of the non-ionized molecule at low pH.

We showed that the extraction with ethyl acetate and the subsequent evaporation process caused a significant (Fig. 5), but reproducible (Table 1), degradation of PB:

- PB sample processing (extraction with ethyl acetate + evaporation) induced spectral changes below 254 nm (Fig. 5A, plots I and II);
- $100 \cdot (\text{slope})_{III}/(\text{slope})_{I}$ (Fig. 5B) corresponded to the overall recovery reported in Table 1: Fig. 5B shows that two different phenomena, i.e. incomplete solvent extraction (plot III vs. plot II) and degradation (plot II vs. plot I, Fig. 5B and C) were responsible for this poor (about 50%) yield. In apparent contrast, the absorbance at 254 nm of the undegraded sample was very similar to that of the degraded one in Fig. 5A but not in Fig. 5B: that can be explained considering that plot II in Fig. 5A corresponded to the spectrum of a mixture of PB and its degradation products [12], which were separated by HPLC and did not contribute to the peak areas of plot III of Fig. 5B.

Furthermore, significant degradation of PB occurred during sample processing but not upon storage of the drug in:

- serum at −20°C, as reported by several authors [5,13,14] and confirmed here by the precision data (Table 1) derived from the analysis of QC aliquots stored at −20°C for several months;
- methanol for 30 h at 22°C or 72 h at 4°C following sample processing as shown in Fig. 5D, in agreement with several other reports [11,14].

Finally, when three adult horses were treated with 4.4 mg/kg of PB, a typical clinical dose [15], the serum levels of the drug and its major metabolite OPB (Fig. 4B and C), were consistent with those reported by Neto et al. [5]. Besides, the chromatograms shown in Figs. 3 and 4A did not show any interference due to endogenous HYD, whose normal levels, are about 100–400 nmol/l, equivalent to about 0.03–0.12 μg/ml, [16–18], and thus too low to be detected by this method.

In conclusion, we developed a simple HPLC assay for the analysis of selected steroidal and non-steroidal drugs, whose sensitivity, despite the occurrence of significant degradation of OPB and PB during the analysis, is sufficient for anti-doping control of racehorses.

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